NEUROPHARMACOLOGICAL, NEUROBIOLOGICAL AND BEHAVIORAL MECHANISMS OF LEARNING AND MEMORY

EDITED BY: Alfredo Meneses, Antonella Gasbarri and Assunta Pompili

PUBLISHED IN: Frontiers in Pharmacology, Frontiers in Neuroscience and Frontiers in Neurology

frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88945-892-9
DOI 10.3389/978-2-88945-892-9

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NEUROPHARMACOLOGICAL, NEUROBIOLOGICAL AND BEHAVIORAL MECHANISMS OF LEARNING AND MEMORY

Topic Editors:
Alfredo Meneses, Centro de Investigación y de Estudios Avanzados (CINVESTAV), Mexico
Antonella Gasbarri, University of L'Aquila, Italy
Assunta Pompili, University of L'Aquila, Italy

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Among the more dynamic topics in science are Neuropharmacological, Neurobiological and Behavioral Mechanisms of Learning and Memory. In this eBook the reader will find fresh reviews and research papers illustrating diverse approaches, which will be seminal in the future.

Citation: Meneses, A., Gasbarri, A., Pompili, A., eds. (2019). Neuropharmacological, Neurobiological and Behavioral Mechanisms of Learning and Memory. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-892-9

Table of Contents

BEHAVIORAL/ENVIROMENTAL

frontiers in Pharmacology

EDITORIAL
published: 15 March 2019
doi: 10.3389/fphar.2019.00218

Editorial: Neuropharmacological, Neurobiological and Behavioral Mechanisms of Learning and Memory

Alfredo Meneses[1]*, *Antonella Gasbarri*[2] *and Assunta Pompili*[2]

[1] *Centro de Investigación y de Estudios Avanzados (CINVESTAV), México City, Mexico,* [2] *Department of Biotechnological and Applied Clinical Sciences, University of L'Aquila, L'Aquila, Italy*

Keywords: drugs, behavior, memory tasks, clinical, humans, animals

OPEN ACCESS

Edited by:
Ashok Kumar,
University of Florida, United States

Reviewed by:
Dong Song,
University of Southern California,
United States

****Correspondence:***
Alfredo Meneses
ameneses@msn.com

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *04 January 2019*
Accepted: *22 February 2019*
Published: *15 March 2019*

Citation:
Meneses A, Gasbarri A and Pompili A (2019) Editorial: Neuropharmacological, Neurobiological and Behavioral Mechanisms of Learning and Memory. Front. Pharmacol. 10:218. doi: 10.3389/fphar.2019.00218

Editorial on the Research Topic

Neuropharmacological, Neurobiological and Behavioral Mechanisms of Learning and Memory

In our initial call, we mentioned that memory is a basic function of the brain, and fundamental in our life. It might be defined according to its content, time, and neurobiological basis: in the former case, as declarative/explicit or non-declarative/implicit memory; regarding time, as short-term (STM) or working, and long-term memory (LTM); and the latter depends on protein and mRNA synthesis. We know now that based on its molecular changes memory, covers several phases and times (e.g., Izquierdo et al., 2006; Ben-Yakov et al., 2015; Asok et al., 2018). According with Asok et al. (2018) there has been important advances in identifying the electrophysiological, genetic, proteomic, and epigenetic underpinnings of long-term memory (LTM).

As the present Research Topic shows, the investigation of memory mechanisms and related brain areas represent one of the most important topics in neuroscience. Memory is a field of scientific investigation in constant expansion. It is unsurprising that thousands of papers already have been published dealing with this subject and we frequently find them in diverse journals, making difficult the identification of clear insights. An effective way to provide appropriate empiric and conceptual frames might be to make available compilations. With this aim, we are organizing the present Research Topic. Certainly, the exact mechanisms of memory remain promising subjects. Readers from preclinical to clinical backgrounds will find interesting neuropharmacological, neurobiological, and/or behavioral insights of the mechanisms of learning and memory, and more importantly contributions combining these approaches. Although we miss theoretical and historical analyses, the richness and variability of tools and approaches used in the papers might reveal key insights and will be a decisive step forward in this topic.

Moreover, attempting going beyond the "memory disorders" notion, the classic Alzheimer's disease and the present dominant behavioral memory tasks (see e.g., Arakawa and Iguchi, 2018) as was the Morris water maze, and considering that cognitive dysfunction occurs in diverse psychiatric disorders (e.g., Millan et al., 2012). For instance, what treatments might be useful for memory alterations component present in posttraumatic stress disorder (PTSD), Attention deficit/hyperactivity disorder (ADHD) and drug addiction? As it becomes clear that Neuropharmacological, Neurobiological and Behavioral Mechanisms are involved, then behavioral, enrichment environmental, and pharmacological manipulations will be necessary?

Although we are far away to our initial aim, however in this Ebook readers will find 36 papers covering original research papers and a few reviews using diverse behavioral memory tasks and approaches. We appreciate very much all authors and reviewers who were generous with their time. A few manuscripts were rejected, sometimes based on fair professionals comments. Almost all the times the editorial office worked hard to get a fair and balance decisions.

AUTHOR CONTRIBUTIONS

AM and AG organized and worked as referee. AP support as referee in several papers.

ACKNOWLEDGMENTS

We appreciate the time and dedication of authors, referees, and editorial office.

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frontiers in Pharmacology

REVIEW
published: 30 June 2017
doi: 10.3389/fphar.2017.00438

The Neuroanatomical, Neurophysiological and Psychological Basis of Memory: Current Models and Their Origins

Eduardo Camina[1,2] *and Francisco Güell*[1]*

[1] *Mind-Brain Group: Biology and Subjectivity in Philosophy and Contemporary Neuroscience, Institute for Culture and Society, University of Navarra, Pamplona, Spain,* [2] *Department of Learning and Curriculum, Faculty of Education and Psychology, University of Navarra, Pamplona, Spain*

This review aims to classify and clarify, from a neuroanatomical, neurophysiological, and psychological perspective, different memory models that are currently widespread in the literature as well as to describe their origins. We believe it is important to consider previous developments without which one cannot adequately understand the kinds of models that are now current in the scientific literature. This article intends to provide a comprehensive and rigorous overview for understanding and ordering the latest scientific advances related to this subject. The main forms of memory presented include sensory memory, short-term memory, and long-term memory. Information from the world around us is first stored by sensory memory, thus enabling the storage and future use of such information. Short-term memory (or memory) refers to information processed in a short period of time. Long-term memory allows us to store information for long periods of time, including information that can be retrieved consciously (explicit memory) or unconsciously (implicit memory).

Keywords: explicit memory, sensory memory, implicit memory, long-term memory, short-term memory

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico
Carlos Alberto Blanco, Comillas Pontifical University, Spain
Elif Engin, McLean Hospital, United States
Francis Bambico, Centre for Addiction and Mental Health, Canada

***Correspondence:**
Francisco Güell
fguell@unav.es

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *13 February 2017*
Accepted: *19 June 2017*
Published: *30 June 2017*

Citation:
Camina E and Güell F (2017) The Neuroanatomical, Neurophysiological and Psychological Basis of Memory: Current Models and Their Origins. Front. Pharmacol. 8:438. doi: 10.3389/fphar.2017.00438

INTRODUCTION

A life full of unconnected events, of errors that do not lead to any lessons and of emotions without the ability to remember them is no life at all. Memory is precisely the capacity that allows us to connect experiences, learn and make sense of our lives. In short, it allows us to build our story. The full range of this complex capacity's neuroanatomical, neurobiological, neurophysiological, and psychological mechanism remain unknown and it presents a challenge for psychologists and neuroscientists who try to explain it. This review attempts to provide a rigorous overview that permits anyone who wants to approach the latest scientific findings on memory to do so, as well as to understand them and properly order them. We will focus on neuroanatomical, neurophysiological, and psychological mechanisms of the different types of memory.

Although knowledge of molecular mechanisms is important for constructing a complete vision of memory models, in this article we can only point out general traits as summarized in this introduction [for more information see (Kandel et al., 2014)]. In addition, knowledge gained from neuroimaging studies (Binder and Desai, 2011), as well as knowledge of the neural markers associated with memory (Meneses, 2015), will likely play a key role in future models of memory mechanisms, but in this review, as stated above, we focus mainly on neuroanatomical, neurophysiological, and psychological mechanisms.

We believe it is important to consider previous developments without which one cannot adequately understand the classifications of memories and the kinds of memory models that are now current in the scientific literature.

The three major classifications of memory that the scientific community deals with today are as follows: sensory memory, short-term memory, and long-term memory. Information from the world around us begins to be stored by sensory memory, making it possible for this information to be accessible in the future. Short-term memory refers to the information processed by the individual in a short period of time. Working memory performs this processing. Long-term memory allows us to store information for long periods of time. This information may be retrieved consciously (explicit memory) or unconsciously (implicit memory).

As Squire (2004) points out, the first theoretical approaches relevant to current neuroscience come from the 19th century. These include Maine de Biran (1804/1929) (Maine de Biran, 1929) who, at the beginning of the century, wrote of mechanical memory, sensitive memory, and representative memory. The philosopher James, and his book *The Principles of Psychology* (James, 1890), is also especially worth highlighting. Therein, James distinguishes between primary and secondary memory, thereby referring to short- and long-term memory, respectively.

The importance of Pavlov (1927) and Fitts and Posner (1967) are especially noteworthy during the first two thirds of the 20th century. Pavlov's studies are related to a type of memory that later would be called associative memory. Meanwhile, Fitts and Posner's studies are considered the first model to explain procedural memory.

Prior to the 60's, most systematizations of memory distinguished a more mechanical type of memory related to the acquisition of skills, which is, in turn, related to activity of the intellect. Unlike what followed, debates in this period were mainly philosophical or based on psychological intuition (Ribot, 1881; Korsafoff, 1890).

Beginning in the 1960s, a series of experimental studies on how the brain stores information emerged, using animals and amnesic patients. Within this decade, Milner, Atkinson, and Shiffrin were especially important researchers.

The experimental modern era arguably began when Milner (1962) demonstrated, with HM experiments, that a seriously ill patient could acquire a new skill (hand-eye coordination) without any memory of having encountered the task before. "While this finding showed that memory is not unitary, discussions at the time tended to set aside motor skills as a special case representing a less cognitive form of memory. The suggestion was that the rest of memory is of one piece and is dependent on medial temporal lobe structures" (Squire and Wixted, 2016).

A few years later, Atkinson and Shiffrin (1968) proposed a modal model of memory that constitutes one of the most influential explanations for the existence of different components in the memory system. The importance of this model is such that it must be explained in the next section, but for now it should simply be mentioned that the modal model establishes the existence of short-term storage (ACP), which receives sensory information that is processed by sensory and data storehouses within long-term memory. This storage system can generate reasoning and new deductions from existing ones.

In the seventies, Tulving, Baddeley, and Hitch and Kandel's investigations are especially noteworthy. Tulving (1972) first proposed the distinction between episodic memory and semantic memory. Baddeley and Hitch (1974) conducted research on the components of working memory. Both authors considered working memory as a limited capacity system that allows temporary storage and manipulation of information necessary to perform complex tasks such as understanding, learning, and reasoning. As explained later on, at first (1974), they proposed the existence of three subsystems within the multi-storehouse model of short-term memory: the central executive, a phonological or articulatory loop and a visuospatial sketchpad. Later, Baddeley (2000) included a fourth subsystem, the episodic buffer, which combines information from the subsystems in a form of temporal representation. Kandel (1976) proposed a model to explain the mechanism of operation in habituation and sensitization. To do this, he used the notion of non-associative memory, which, as we shall see, is one of the four types of non-declarative or implicit memory, like that which refers to new behaviors learned through repeated exposure to a single stimulus. According to Kandel, new behaviors can be classified into two processes: sensitization and habituation. On the one hand, for habituation, acetylcholine is progressively consumed, decreasing the effectiveness of the stimulus and thereby the motor response. On the other hand, the presence of serotonin in sensitization, secreted by another sensory nerve terminal, causes an excess of acetylcholine. An enhanced motor response thus emerges.

In the 1980s, the differences between declarative and non-declarative memories were consolidated and disseminated. This, together with contributions from Tulving and others, such as Di Lollo or Graf and Schacter, enabled a more precise classification of different types of memory. To date, Di Lollo's model of iconic memory (Di Lollo, 1980) has been the most widely accepted and studied of the three existing types of sensory memory. As discussed in the next section, Di Lollo considered iconic memory a storage unit consisting of two components: the persistence of vision and information. Graf and Schacter (1985) proposed a general difference between declarative memory (explicit) and non-declarative memory (implicit/procedural). This stems from the distinction that Tulving (1972) proposed between the aforementioned episodic memory and semantic memory (both, as we will see, are currently included in declarative memory).

In the 90's, a classification of the types of memory emerged, but the way they act and their interrelation was still unclear. In order to clarify its operation, Packard and McGaugh (1996) proposed that memory systems operate independently and in parallel. For example, an adverse event in childhood (e.g., seeing your grandfather being run over by a combine) can, on the one hand, consolidate as a stable declarative memory for the event itself (the sound of a combine always makes you

remember that moment-episodic memory) and, on the other hand, can crystallize in non-declarative memory and result in a phobia experienced as a personality trait rather than as a mere memory (being near a combine will always produce panic and induces a desire to escape that situation-associative memory). Several authors (Tulving et al., 1982) had already mentioned the idea of priming as a separate type of memory, but it was not until the 90's that experiments were conducted to show it (Hamann and Squire, 1997; Stark and Squire, 2000; Levy et al., 2004). These studies show that severely amnesic patients can exhibit completely intact priming while performing memory tests that include conventional recognition of the same test items (Squire, 2004).

Thanks to the development of new 21st century technologies, researchers have been able to more accurately locate brain areas that are associated with different types of memory. Although this pertains to topics to be addressed in detail in the next section, there are two examples that we consider significant to the application of these new techniques and the significant progress made in understanding memory storage. On the one hand, Ergorul and Eichenbaum's experiment (Ergorul and Eichenbaum, 2004) shows that animals are able to remember the "context in which they experienced specific stimuli, and that this capacity also depends on the hippocampus" (Dickerson and Eichenbaum, 2010). This process is closely related to the formation of episodic memory. On the other hand, neuroimaging studies that Binder and Desai (2011) conducted show "two striking results: the participation of modality-specific sensory, motor, and emotion systems in language comprehension, and the existence of large brain regions that participate in comprehension tasks but are not modality-specific." With this in mind, Binder and Desai (2011) claims that semantic memory consists of two representations, including a specific mode and a supramodal mode. Again, this will be explained in more detail in what follows.

The research of the cellular and molecular substrates of memory has received much attention since Lomo (1966) described in the 60's "a cellular model of experience-dependent plasticity—long-term potentiation (LTP)" (Kandel et al., 2014). According to Lisman et al. (2012): "LTP is a process whereby brief periods of synaptic activity can produce a long-lasting increase in the strength of a synapse, as shown by an increase in the size of the excitatory postsynaptic current." NMDA receptors are double-gated, as their activation requires both postsynaptic membrane depolarization as well as presynaptic release of glutamate. Once activated by these conditions, NMDA receptors trigger a strong postsynaptic influx of Ca2+ that induce LTP through a variety of pathways including CaMKII, PKC, PKA, and MAPK (Kandel et al., 2014).

With this brief historical and conceptual introduction laid out, we intend to delve into different types of memory in order to present the models that the scientific community has most accepted thus far. In the last section, and before the glossary, we identify the likely directions for future research. Now we turn on to our main task, presenting an overview of the latest scientific findings on memory, classified according to different types and mechanisms.

SENSORY MEMORY: ICONIC MEMORY

"Sensory memory is the capacity for briefly retaining the large amounts of information that people encounter daily" (Siegler and Alibali, 2005). There are three types of sensory memory: echoic memory, iconic memory, and haptic memory. Iconic memory retains information that is gathered through sight, echoic memory retains information gathered through auditory stimuli and haptic memory retains data acquired through touch.

Scientific research has focused mainly on iconic memory; information on echoic and haptic memory is comparatively scarce. Thus, taking into account the goals of this article and that it is aimed at a higher education audience, presenting iconic memory as a paradigm of sensory memory is sufficient for an introductory overview.

Iconic memory retains information from the sense of sight with an approximate duration of 1 s. This reservoir of information then passes to short-term vision memory (which is analogous, as we shall see shortly, to the visuospatial sketchpad with which working memory operates).

Di Lollo's model (Di Lollo, 1980) is the most widely accepted model of iconic memory. Therein, he considered iconic memory a storehouse constituted by two components: the persistence of vision and information.

(a) Persistence of vision. Iconic memory corresponds to the pre-categorical representation image/visual that remains between 100 and 300 ms. It is sensitive to physical parameters, such that it depends on retinal photoreceptors (rods and cones). It also depends on various cells in the visual system and on retinal ganglion cells M (transition cells) and P (sustained cells). It concludes its representation in the primary visual cortex (V1) of the occipital lobes. "The occipital lobe is responsible for processing visual information" (Kamel and Malik, 2014).

(b) Persistence of information. Iconic memory is a storehouse of information that lasts 800 ms and that represents a codified and already categorized version of the visual image. It plays the role of storehouse for post-categorical memory, which provides visual short-term memory with information to be consolidated. For this, it travels through the ventral route (V) (V1 → V2 → V5 → inferior temporal cortex).

Subsequent research on visual persistence from Coltheart (Coltheart, 1983) and Sperling's studies (Sperling, 1960) on the persistence of information led to the definition of three characteristics pertaining to iconic memory: a large capacity, a short duration, and a pre-categorical nature.

Sperling (1960) demonstrated this large capacity after presenting the results of his total and partial reports. The full report consisted in presenting a 3 × 3 or 3 × 4 matrix of alphanumeric characters for a short period of time to subjects and later asking them which characters they remembered. On the other hand, in the partial report, subjects were directed to remember the characters in a row specifically assigned to them in the instructions. The total report's results showed that subjects were only able to recall between 3 or 4 letters of the total number. However, in the partial report, subjects remembered around 75% of those that were asked. In extrapolating the partial report's data to the total, it follows that individuals could report 9 of the

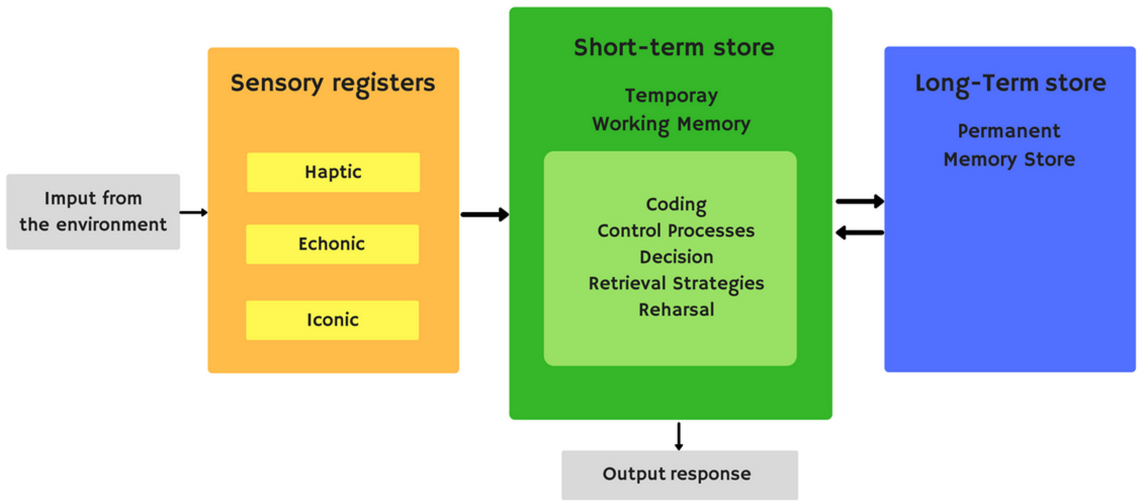

FIGURE 1 | Atkinson and Shiffrin's modal model.

12 letters contained in the instructions (80% of the total), thus demonstrating a large capacity.

Regarding short-term, Sperling interpreted the results of the partial report as due to the rapid decline of the visual sign and reaffirmed this short duration by obtaining a decrease in the number of letters reported by the subject in delaying the audio signal for choosing a row to remember in the presentation. Averbach and Coriell's experiments (Averbach and Coriell, 1961) corroborated Sperling's conclusion; they presented a variety of letters for a certain period of time to the subject. After each letter, and in the same position, they showed a particular visual sign. The participant's task was to name the letter that occupied the position of the visual sign. When the visual sign appeared immediately after the letters, participants could correctly name the letter that occupied the position of the sign, however, as the presentation of the sign became more delayed, participant performance worsened. These results also show the rapid decline of visual information.

Finally, regarding its pre-categorical nature, Sperling considered the information contained in this storehouse as physical information that maintains the raw data that is not related to the meaning of stimuli. Subsequently, evidence has been obtained that this system is not entirely pre-categorical (Loftus et al., 1992) since the task improves when the stimuli to remember are letters or numbers instead of meaningless forms.

SHORT-TERM MEMORY

Short-term memory is the ability to keep a small amount of information available for a short period of time. Atkinson and Shiffrin's modal model (Atkinson and Shiffrin, 1968) is one of the most influential explanations for the existence of different components in the memory system (**Figure 1**). This model has some similarities with Broadbent's previous model (Broadbent, 1958). The modal model establishes the existence of a short-term storehouse with limited capacity. The short-term storehouse receives sensory information processed by sensory storehouses and data in long-term memory. In addition, the short-term storehouse can also send information to the structures involved in long-term memory. This storehouse can generate reasoning and new deductions from existing ones. This model implies that the short-term storehouse functions as a kind of working memory, a system to retain and manipulate information temporarily as part of a wide range of essential cognitive tasks such as learning, reasoning, and understanding. They, in turn, give short-term storage central importance in the overall processing of information by attributing to it the role of controlling the executive system, responsible for the coordination and control of many complex subroutines in charge of acquiring new material and recovering old material in long-term storage.

Despite the explanatory power of Atkinson and Shiffrin's model, there were a number of issues that this model could not resolve, causing criticism of it. For example, this model implies that the longer an item remains in memory, the more likely it is to be transferred to long-term storage. But studies like those of Tulving and Pearlstone (1966) and Craik and Watkins (1973) show that said relationship does not exist.

Given these criticisms, new models began to appear to explain memory, such as those from Cowan (1988, 1995, 1999) and Goldman-Rakic (1995). Among them, Craik and Lockhart's process model (Craik and Lockhart, 1972) and Baddeley and Hitch's structural model (Baddeley and Hitch, 1974) were the most prominent; the latter is the most commonly accepted one today and thus we will focus on it in this article.

As an introduction, it can be argued that Craik and Lockhart (1972) understood memory not as a process through which information is deepened at higher levels until it becomes part of long-term memory, but rather as a system of storehouses. Despite an emphasis on information processing (instead of structure), they continued to accept the existence of short-term memory as independent from long-term memory. For their part, Baddeley

and Hitch's proposal (Baddeley and Hitch, 1974) contemplated a multi-component working memory instead of a storage unit in the short term.

Working Memory

"The term working memory refers to a brain system that provides temporary storage and manipulation of the information necessary for such complex cognitive tasks as language comprehension, learning and reasoning" (Baddeley, 1992). At first (1974), they proposed the existence of three subsystems within the multi-storehouse model of short-term memory: the central executive, a phonological or articulatory loop and a visuospatial sketchpad.

In general, we can say that the central executive controls attention, "the phonological loop ensures retention of verbal information and the visuospatial sketchpad is responsible for storage visual and spatial information" (Grigorenko et al., 2012). The latter two sub-memory systems are equivalent to verbal and visual short-term memory systems, respectively.

Wang and Bellugi (1994) presented a genetically based test that supports the functional and anatomical separation of Baddeley's model with phonological and visuospatial storehouses. They compared two genetic syndromes (Williams and Down) with different brain morphology. Williams syndrome patients, despite having widespread mental handicaps, preserve their language skills, while Down syndrome patients preserve more partial capacities, but have very limited language skills. It was therefore assumed that the former would be better at verbal tasks related to operative memory, and that the latter would be better at visuospatial tasks related to operative memory. As expected, subjects with Williams syndrome performed better at phonological tasks, while subjects with Down syndrome, in turn, performed better at spatial tasks.

Later, Baddeley (2000) included a fourth subsystem, the episodic buffer (**Figure 2**), which combines information from the different subsystems in a kind of temporal representation.

Here we will focus on the different subsystems that make up Baddeley's multi-storehouse model (2000), i.e., the central executive, the phonological or articulatory loop, the visuospatial sketchpad and the episodic buffer.

The Central Executive

The central executive is a system of attention control with limited processing capacity. Baddeley (1986) adopted a model originally proposed by Norman and Shallice (1986), in which actions are controlled in two ways. "Behavior that is routine and habitual is controlled automatically by a range of schemas, well-learned processes that allow us to respond appropriately to the environment" (Baddeley and Hitch, 2010). Processes that are not recognized as habitual are controlled by a second system, the supervisory attention system. This system uses long-term knowledge to propose novel behavioral solutions and to weigh options before deciding on a response.

In its original version, the central executive was considered an overall system capable of processing and storing. However, Baddeley and Logie (1999) proposed that it only has attention capacity.

Subsequent studies have proposed to complement the executive system with the episodic buffer as other separate storage system: "the episodic buffer clearly does represent a change within the working memory framework, whether conceived as a new component, or as a fractionation of the older version of the central executive"(Baddeley, 2000).

Baddeley and Logie understand the central executive as the result of the integration of several processes: the ability to focus attention, the ability to divide attention between two or more tasks, and the ability to control long-term memory access (Baddeley et al., 1991; Logie et al., 2004; Baddeley, 2007). The way to accomplish this may be with one or more types of inhibition (Engle et al., 1999; Miyake et al., 2000). This approach accepts that the frontal lobes play an important role in executive control, although there are differing opinions on the functions' precise location (Duncan and Owen, 2000; Shallice, 2002).

Visuospatial Sketchpad

It has been suggested that the sketchpad's main function is to create and maintain a visuospatial representation that persists through the irregular form found in eye movement and that characterizes our exploration of the visual world (Luck, 2007).

It has been shown that spatial tasks such as driving a car can interfere with spatial skills, while exclusively visual tasks, such as watching a series of images or colored shapes, can interfere with the recall of objects or shapes (Logie, 1986; Klauer and Zhao, 2004). These patterns of interference, together with cases of brain-damaged patients that show a deficit in one kind of task but not the other (Della Sala and Logie, 2002), suggest that spatial information and visual characteristics can be stored separately.

The visuospatial sketchpad seems to involve a number of areas, predominantly in the brain's right hemisphere. On the one hand, it contains a visual component that reflects the processing and storage of objects and their visual features. On the other hand, it contains a second parietal area, presumably involved in spatial aspects.

Phonological Buffer

It can be argued that the phonological buffer supports language acquisition by providing the ability to store new words, while they are consolidated into long-term memory (Baddeley et al., 1998). Within this phonological loop, two basic sub-processes emerge: a short-term acoustic storehouse and a subvocal articulatory rehearsal process. The existence of the former is indicated by the effect of phonological similarity, where speech is less accurate when repeating "similar-sounding words such as MAN CAP CAT MAT CAN, than dissimilar words such as PIT DAY COW PEN TOP. Similarity of meaning (HUGE LARGE BIG WIDE TALL) has little effect on immediate recall. On the other hand if several trials are given to learn a longer list of say 10 words, meaning becomes all-important and sound loses it power, consistent with different systems for short-term and long-term storage (Baddeley, 1966a,b). Evidence for the importance of rehearsal comes from the word length effect, whereby immediate recall of long words (e.g., REFRIGERATOR UNIVERSITY TUBERCULOSIS OPPORTUNITY HIPPOPOTAMUS) is much more error-prone than for short words (Baddeley et al., 1975)" (Baddeley and

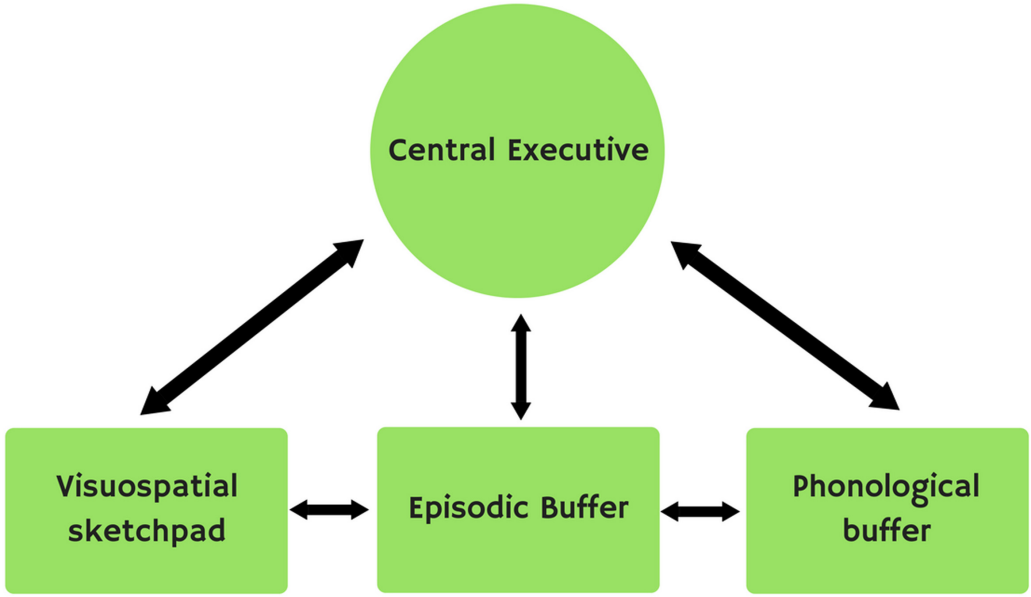

FIGURE 2 | Baddeley's model.

Hitch, 2010). Baddeley and Hitch (1974) proposed that retention of items in the short-term storehouse quickly fade, but can be maintained by repeating them.

With respect to cerebral location, the phonological loop is found in the brain's left hemisphere "The loop is assumed to hold verbal and acoustic information using a temporary store and an articulatory rehearsal system, which clinical lesion studies, and subsequently neuroradiological studies, suggested are principally associated with Brodmann areas, 40 and 44, respectively" (Baddeley, 2000).

Episodic Buffer

The verbal and visual systems within the conventional model of working memory may explain many aspects, but Baddeley (2000) points out that evidence from patients with short-term memory deficits— who resist memorizing prose (with a verbal span much higher than that of isolated words) and resist serial memory of articulatory suppression— leads to supposing that a storehouse of additional support exists. This is seen in the existence of a new mechanism that combines information from multiple subsystems into a form of temporal representation. Baddeley (2000) proposed the term episodic buffer for this new kind of representation.

The episodic buffer is thus a temporary storage system capable of integrating information from different sources, likely controlled by the central executive. "The buffer is episodic in the sense that it holds episodes whereby information is integrated across space and potentially extended across time" (Baddeley, 2000). It can be preserved in patients with advanced amnesia and severe impairment of long-term episodic memory.

With that said, it is possible to consider the episodic buffer as conceptual short-term memory. Studies to date do not specify activity in a specific area. As Potter (1999) said: "The conceptual short-term memory hypothesis proposes that when a stimulus is identified, its meaning is rapidly activated and maintained briefly in conceptual short-term memory."

LONG-TERM MEMORY

Long-term memory refers to unlimited storage information to be maintained for long periods, even for life. There are two types of long-term memory: declarative or explicit memory and non-declarative or implicit memory.

Explicit memory refers to information that can be consciously evoked. There are two types of declarative memory: episodic memory and semantic memory. For its part, implicit memory encompasses all unconscious memories, such as certain abilities or skills. There are four types of implicit memory, including procedural, associative, non-associative, and priming.

Declarative/Explicit Memory

Explicit memory refers to information that can be evoked consciously. There are two types of declarative memory: episodic memory and semantic memory. As shown below, episodic memory stores personal experiences and semantic memory stores information about facts.

Episodic Memory

"Episodic memory involves the ability to learn, store, and retrieve information about unique personal experiences that occur in daily life. These memories typically include information about the time and place of an event, as well as detailed information about the event itself." (Dickerson and Eichenbaum, 2010).

There are a number of neural components that are closely related to the proper functioning of episodic memory, which

include the following: the cortex near the hippocampus [as discussed below, the perirhinal cortex (PRC), the entorhinal cortex, and the parahippocampal cortex (PHC)], cortical and subcortical structures, and the circuits within the medial temporal lobe and hippocampus.

The cortices near the hippocampus extensively interact with a number of cortical and subcortical structures; cortical components have key roles in various aspects of perception and cognition, while the medial temporal lobe structures mediate the organization and the persistence of the memory network, whose data is stored in these cortical areas (Dickerson and Eichenbaum, 2010).

The structures directly related to the hippocampus include the entorhinal, the parahippocampal, and the perirhinal cortices. Each one is discussed in detail below.

The entorhinal cortex is the main interface between the hippocampus and neocortex, thus it is associated with the distribution of information to and from the hippocampus. The surface layers (II and III) of the entorhinal cortex project out toward the dentate gyrus and hippocampus. While layer II mainly projects out toward the dentate gyrus and the CA3 region of the hippocampus, layer III mainly projects out toward the hippocampal CA1 region and the subiculum. These layers receive input signals from other cortical areas, particularly the association cortices, the PRC and the parahippocampal gyrus, as well as the prefrontal cortex. Layers II and III receive highly processed inputs from each sensory modality, and inputs related to ongoing cognitive processes. Deep layers, particularly layer V, receive one of the three output signals from the hippocampus and, in turn, exchange connections with other cortical areas that project out toward the superficial entorhinal cortex.

The PRC has a role in visual object recognition, while the PHC is involved in the perception of the local environment and processing information related to that place. Thus, fMRI studies indicate that the PHC becomes very active when human subjects receive topographical stimuli such as landscapes or rooms. Epstein and Kanwisher (1998) first described the PHC and Aguirre et al. (1996, 1998) and Ishai et al. (1999) later backed up that description.

Finally the hippocampus is responsible for the formation and retrieval of memories. That is, the information that the three cortices described above process reach the hippocampus where new memories are generated and from which they can later be retrieved. Episodic memory recall involves a spatial and temporal context of specific experiences. For further review of the mechanisms of memory formation see Craver (2003).

As Dickerson and Eichenbaum (2010) point out in their review, "several investigators have argued that animals are indeed capable of remembering the context in which they experienced specific stimuli, and that this capacity also depends on the hippocampus." Ergorul and Eichenbaum (2004) published a significant study to this effect in which they developed a series of tasks for rats to assess their memory of events, which combined an odor (what), the place of the experience (where), and the relation to other experiences (when). The rats were presented with a sample of an odor in one specific place along the edge of a large open field. Subsequently, as a way of testing their memory, they were presented with a choice between two arbitrarily selected odors in their original locations. The results of the test showed that normal rats use a combination of where and what information to judge the timing of the events, while rats with a damaged hippocampus cannot manage to effectively combine what, when, and where information in order to form a recovered memory.

Three years later Eichenbaum et al. (2007) proposed a functional organization of memory's medial temporal lobe system: "Neocortical input regarding the object features ("what") converges in the PRC and lateral entorhinal area (LEA), whereas details about the location ("where") of objects converge in the PHC and medial entorhinal area (MEA). These streams converge in the hippocampus, which represents items in the context in which they were experienced. Reverse projections follow the same pathways back to the parahippocampal and neocortical regions"(Eichenbaum et al., 2007).

It should be noted that memory of faces is typically associated with activity in the perirhinal and hippocampus rostral regions, while memory of objects is typically associated with wider-ranging activity (Preston et al., 2010).

Both results concerning functional an anatomic and characterizations in animal models are consistent with the hypothesis that is guided by anatomic criteria about the functional organization of the hippocampal system (Dickerson and Eichenbaum, 2010).

"The ventral temporal cortex, including fusiform gyrus, is commonly engaged when pictures of visual objects are presented, and the lateral temporal cortex including superior temporal gyrus is typically engaged during the encoding of auditory information" (Dickerson and Eichenbaum, 2010).

Semantic Memory

As noted, in the context of long-term memory, there were two types of memory, corresponding to declarative and non-declarative memory. Within declarative memory, we find both episodic memory, as discussed above, and semantic memory, as discussed below.

Human beings have the ability to represent concepts in language. This ability allows us not only to disseminate conceptual knowledge to others, but also to manipulate, associate, and combine these concepts. Therefore, as Binder and Desai shows, "humans use conceptual knowledge for much more than merely interacting with objects. All of human culture, including science, literature, social institutions, religion, and art, is constructed from conceptual knowledge" (Binder and Desai, 2011). Activities such as reasoning, planning for the future or reminiscing about the past depend on the activation of concepts stored in semantic memory (Mahon and Caramazza, 2008).

Binder and Desai showed two striking results related to neuroimaging research: on the one hand, the participation of the specific sensory, motor and emotional modality in understanding language and, on the other hand, the existence of large regions of the brain (the inferior parietal lobe and a large part of the temporal lobe) involved in tasks related to understanding. These latter regions converge on the many currents involved in perception processing, and these convergences allow supramodal

representations of perceptual experience that support a variety of conceptual functions, including language, social cognition, object recognition, and the extraordinary human ability to remember the past and imagine the future (Binder and Desai, 2011). Therefore, accepting their argument, semantic memory consists of two representations: a specific modality and supramodal modality.

In this regard, Binder and Desai found several objections. A not inconsiderable one is that activations observed in imaging experiments could be an epiphenomenon rather than causally related to understanding. Therefore, the involvement of the motor system for processing a text would contribute to understanding and is not a mere product. Another critical point is the possibility of interpreting that collected activations represent images after understanding takes place. However, and as they showed in their review (Binder and Desai, 2011), in studies of neuroimaging with high temporal resolution, the activation of motor regions during the processing of a text appears to be rapid, about 150–200 ms after each word (Pulvermüller et al., 2005; Boulenger et al., 2006; Hoenig et al., 2008; Revill et al., 2008).

"These converging results provide compelling evidence that sensory-motor cortices play an essential role in conceptual representation. Although it is often overlooked in reviews of embodied cognition, emotion is as much a modality of experience as sensory and motor processing (Vigliocco et al., 2009). Words and concepts vary in the magnitude and specific type of emotional response they evoke, and these emotional responses are a large part of the meaning of many concepts" (Binder and Desai, 2011).

Following Binder and Desai, brain appears to use supramodal abstract representations for conceptual tasks. In this regard, it can be convincingly argued that the human brain has large areas of cortex that are between the sensory systems and motor modalities and, therefore, Damasio's idea convergence zones seems plausible (Damasio, 1989). "These heteromodal areas include the inferior parietal cortex (angular and supramarginal gyri), large parts of the middle and inferior temporal gyri, and anterior portions of the fusiform gyrus (Mesulam, 1985)" (Binder and Desai, 2011).

A second argument supporting the hypothesis that the brain appears to use supramodal abstract representations during conceptual work comes from patients with damage to the lower and lateral temporal lobe. The clinical profile of semantic dementia is marked by progressive atrophy in the temporal lobe and loss of multimodal semantic memory (Hodges et al., 1992; Mummery et al., 2000). Patients with semantic dementia is characterized by a loss of conceptual knowledge, and this loss may reflect the disruption of a central semantic hub or the degeneration of a temporosylvian language network for verbal concepts (Irish et al., 2014). These patients manifesting in striking alterations in naming and comprehension (Irish et al., 2016). These patients are "characterized by a clear dissociation between marked single-word comprehension" (Agosta et al., 2010), unable to retrieve the names of objects, irregular word reading deficits, identify the color the correct objects, and sparing of fluency, phonology, syntax and working memory (Binder and Desai, 2011).

Basically, these deficits do not seem to be categorical, constituting further evidence that semantic impairment does not imply strongly modal representations and, therefore, the modular and supramodal systems are presented as an interactive continuum of hierarchically ordered neuronal combinations, supporting representations that are progressively more idealized and combined (Binder and Desai, 2011). These systems correspond to Damasio's idea of areas of local convergence and with Barsalou's idea of systems of unimodal perceptual symbols (Lambon Ralph et al., 2007). In addition to bottom-up input within their associated modality, each system receives top-down input from other modal and attention systems. These systems are modal in the sense that their output is a analogic or isomorphic representation of the information that they receive bottom-up within their associated modality (Barsalou, 1999a).

As observed by Binder and Desai: "These modal convergence zones then converge with each other in higher-level cortices located in the inferior parietal lobe and much of the ventral and lateral temporal lobe (. . .). One function of these high-level convergences is to bind representations from two or more modalities, such as the sound and visual appearance of an animal, or the visual representation and action knowledge associated with a hand tool (Wernicke, 1974; Damasio, 1989; Barsalou, 1999b; Patterson et al., 2007). Such supramodal representations capture similarity structures that define categories, such as the collection of attributes that place 'pear' and 'light bulb' in different categories despite a superficial similarity of appearance, and 'pear' and 'pine-apple' in the same category despite very different appearances (Rogers and McClelland, 2004). More generally, supramodal representations allow the efficient manipulation of abstract, schematic conceptual knowledge that characterizes natural language, social cognition, and other forms of highly creative thinking (Dove, 2011; Diefenbach et al., 2013)" (Binder and Desai, 2011).

Non-declarative/Implied Memory

As noted, long-term memory refers to unlimited information storage that can be maintained for long periods, even for life. There are two types of long-term memory: declarative or explicit memory and non-declarative or implied memory.

Implicit memory encompasses all unconscious memories, as well as certain abilities or skills. There are four types of implicit memory: procedural, associative, non-associative, and priming. Each one is detailed below.

Procedural Memory: Habits and Skill

Procedural memory is the part of memory that participates in recalling motor and executive skills that are necessary to perform a task. It is an executive system that guides activity and usually works at an unconscious level. When necessary, procedural memories are retrieved automatically for use in the implementation of complex procedures related to motor and intellectual skills.

Development of these rote capacities occurs through procedural learning, that is, by systematically repeating a complex activity until acquiring and automatizing the capacity

of all neural systems involved in performing the task to work together.

The acquisition of skills requires practice. However, the simple repetition of a task does not ensure skill acquisition. A skill is thought to be acquired when behavior changes as a result of experience or practice. This is known as learning and it is not a directly observable phenomenon. Here we will discuss two models for acquiring skills.

The first model comes from Fitts's team (Fitts, 1954; Fitts and Posner, 1967). These scientists propose an explanatory model of skill acquisition, based on the idea of learning as a process in three phases:

(a) Cognitive phase: The process begins with the acquisition of knowledge about the factors that make up a particular observed behavior. At this point, the psychological process of attention is important. The skill to be acquired must be broken down into its basic components and one must understand how these components are combined to form a whole in the correct execution of the task (Fitts, 1954; Fitts and Posner, 1967).
(b) Associative phase: Individual repeated practice takes place until there is an automatic response pattern. As one progresses through this point, the actions that are important for the implementation of a skill are learned and become automated, just as any superfluous or ineffective actions disappears. The individual sensory system acquires the exact symbolic and spatial data required for the appropriate execution of the skill (Fitts, 1954; Fitts and Posner, 1967).
(c) Autonomous/procedural phase: This is the final phase and it consists in perfecting acquired skills. The ability to judge which stimuli are important and unimportant improves and a lower level of conscious thought is required because the skill becomes automated (Fitts, 1954; Fitts and Posner, 1967).

The other model corresponds to (Tadlock, 2005) and is called Predictive Cycle. This model proposes that learning only requires conscious maintenance of the desired end result. The model consists of the following phases: Trial, error, implicit result analysis, and decision-making at the implicit level of the way in which execution of the next test must be changed for successful implementation. These steps are repeated again and again until the subject builds or remodels his/her neural network so that it can guide the activity without the need for conscious thought.

A number of factors are involved when acquiring and implementing skills, including attention and pressure. For the acquisition of a new skill one must pay attention to the steps to be undertaken. This process involves using working memory to allow for connecting the different steps involved. Procedural memory acquires the habit with the help of the attention span, but it implies a lesser performance. However, with practice, procedural knowledge is developed. Procedural knowledge operates away from working memory, which allows for the implementation of the most automated skills (Anderson, 1993). Meanwhile, pressure can affect the performance of a task in two ways: choking or clutchness. The choking phenomenon occurs when experienced and skilled performers fail under stress. Auto-focus theories suggest that pressure causes an increase in anxiety and self-consciousness concerning correct execution. This ends up causing increased attention directed toward processes directly involved in the execution of the skill (Beilock and Carr, 2001). On the other hand, the attention span allows the habit to be acquired in refers to giving a top performance on a given task when pressure is highest.

Because they are especially relevant, we will briefly outline brain components involved in the acquisition of new skills and habits, including the basal ganglia, cerebellum and limbic system.

As Christos and Emmanuel explain (Constantinidis and Procyk, 2004), "basal ganglia are formed by several sub-structures: the striatum, the globus pallidus, the substantia nigra, and the subthalamic nucleus." The basal ganglia are a collection of nuclei found on both sides of the thalamus, outside of and around the limbic system, but below the cingulate gyrus and within the temporal lobes. The striatum or striate nucleus is the main gateway for information to the basal ganglia. In turn, the striatum receives information from the cerebral cortex. Essentially, there are two parallel processing paths that depart from the striatum, each of which acts in opposition to each other in the control of movement and enables associations with other relevant functional structures (Beilock and Carr, 2001). Both work together as a neuronal feedback loop. There are many circuits that reach the striatum from other brain areas, including the limbic cortex (associated with emotional processing); the ventral striatum (related to the processing of rewards), and other important motor regions involved in movement (Alexander and Crutcher, 1990). Currently, striatal neuronal plasticity enables basal ganglia circuits to interact with other structures and thereby contribute to the processing of procedural memory (Haber et al., 2000).

The cerebellum is involved in the execution of movements and the perfection of motor agility needed procedural skills. Damage to this area can impede one from relearning motor skills and recent studies have linked it to the process of automating unconscious skills during the learning phase (Kreitzer, 2009).

The limbic system shares anatomical structures with a component of the neostriatum, which assumes primary responsibility for the control of procedural memory. There is a special protein membrane associated with the limbic system that runs through the nucleus basalis. Thus, activation of brain regions that work together during the operation of procedural memory can be followed through the protein membrane associated with the limbic system.

As a final note on procedural memory, whereas earlier theories proposed a passive role whereby memories were shielded from interfering stimuli during sleep (Vertes and Eastman, 2000; Vertes, 2004), current theories suggest a more active role in which memories undergo a process of consolidation during sleep (Ellenbogen et al., 2006). Furthermore, in human beings, this process of consolidation is thought to contribute to the development of procedural knowledge, especially when it occurs right after the initial phase of memory acquisition (Karni et al., 1994; Gais et al., 2000; Stickgold et al., 2000a,b; Saywell and

Taylor, 2008). Within the scope of motor skills related to procedural memory, there is evidence to show that there is no improvement in skills if followed by short NREM sleep (stages 2–4 sleep), such as a short nap (Walker et al., 2002). However, REM sleep (a sleep phase with an increased frequency and intensity of the so-called dream state) followed by a period of slow wave sleep has proven to be the most effective combination for procedural memory consolidation, especially immediately following skill acquisition (Siegel, 2001).

Associative Memory: Classical and Operant Conditioning

Associative memory refers to the storage and retrieval of information through association with other information. The acquisition of associative memory is carried out with two types of conditioning: classical conditioning and operant conditioning. Classical conditioning is associative learning between stimuli and behavior. Meanwhile, operant conditioning is a form of learning in which new behaviors develop in terms of their consequences. Associationist philosophers have also worked with the latter model (Hartley, 1749; Mill, 1829). We will look more closely at both.

The close association between two stimuli over time causes classical conditioning: first a conditioned stimulus and then an unconditioned stimulus. While a conditioned stimulus does not automatically trigger a response, an unconditioned stimulus does just that. By repeating a conditioned stimulus over time before an unconditioned stimulus, a conditioned stimulus acquires characteristics that simulate being necessary for an unconditioned stimulus. Pavlov's Dog (Pavlov, 1927) is a clear example. The dog produces saliva when it detects the presence of food (unconditioned stimulus). If the sound of a bell goes off (conditioned stimulus) during the act of giving the dog food, the dog will associate the sound of the bell with the presence of food. In successively repeating this, the dog will associate the unconditioned stimulus with the conditioned stimulus, thus producing saliva when just hearing the bell.

Although Skinner is considered to be the originator of operant conditioning, his research drew upon Thorndike's law of effect. For operant conditioning, as has already been mentioned, positive consequences following a behavior promote its repetition. Conversely, if the behavior involves negative consequences, the behavior will be repeated less. Thorndike (1932) called this conditioning instrumental because it suggests that the behavior serves as a means to an end and emerges from trial and error. Skinner later coined the term that is now widely associated with this law of effect – reinforcement (Skinner, 1938).

Non-associative Memory: Habituation and Sensitization

Non-associative memory is one of three types of non-declarative or implicit memory and refers to newly learned behavior through repeated exposure to an isolated stimulus.

New behavior can be classified into two processes: sensitization and habituation (Alonso, 2008). Before delving into each process, it is worth noting that the simplicity in acquiring this type of memory has advanced knowledge of the learning process. This is due to the fact that both animals and human beings have these two processes, such that it is very likely that, in this regard, they share a molecular biological basis. Kandel (1976) proposed a model to explain habituation and sensitization's operation mechanism. On one hand, for habituation, acetylcholine is progressively consumed, decreasing the effectiveness of the stimulus and thus the motor response. Furthermore, for sensitization, the presence of serotonin, secreted by another sensory nerve terminal, causes an excess of acetylcholine. Thus an enhanced motor response emerges. Let's look at the two processes that take place in the acquisition of new behaviors— processes that are part of non-associative memory, habituation and sensitization.

Habituation, in this context, is linked to repetition. The repetition of a stimulus leads to a decrease in its response, which is known as habituation. Repeated exposure to a stimulus serves to stop responding to potentially important, but situationally irrelevant stimuli. Habituation could be due to a process of synaptic depression as a result of repeated activation. Thus, habituation is thought to be related to a decrease in the efficiency of synaptic transmission, a decrease that may be caused by a conductivity change in the membrane of the stimulated neuron's iconic channels.

Unlike habituation, sensitization consists in an increase in response to a stimulus due to the repeated introduction thereof. Although the processes that produce sensitization are the same as those that produce habituation, sensitization's effects are the opposite since it results in an increase of the original response. The process of sensitization may be due to a provision in transmission, whether it be presynaptic or postsynaptic.

Priming

Priming, the fourth modality of non-declarative or implicit memory, is an effect whereby exposure to certain stimuli influences the response given to stimuli presented later.

An example is in order. If you present a list of words to a person that contains the word 'ball,' and then the person is asked to participate in a task to complete words, they are more likely to respond with the word ball to the presentation of the word bowl than if they had not previously seen that word in the original list. Thus, the priming capacity can affect the choice of a particular word on a test to complete words, even long after conscious recollection of the primed words has been forgotten.

Another context where this can be seen is in asking a participant to identify an image from a small fragment. The participant is shown a larger portion of the image over time, giving them the ability to identify the image at the end. The participant will take longer to identify the image if it is the first time he/she sees it. But if he/she already saw it in a previous trial, he/she takes less time (Kolb and Whishaw, 2003).

FUTURE DIRECTIONS

In spite of recent progress, a number of important questions remain to be tackled.

Many of these questions have to do with molecular processes of memory consolidation, retrieval, and decay. Take, for instance, the processes underlying the LTP of synaptic strength among neurons of the hippocampus (Dong et al., 2015). In their review, Hardt et al. (2013) point out that while the "molecular processes involved in establishing LTP have been characterized well, the decay of early and late LTP is poorly understood." One possibility that has recently been suggested is that LTP decay is mediated by AMPAR endocytosis, which in turn implies that inhibition of this process could preserve LTP and help to prevent memory loss (Dong et al., 2015).

Other recent work shows the critical role of dopamine as a signal that promotes the stable incorporation of novel information into long-term hippocampal memory (Otmakhova et al., 2013). Indeed, dopamine neurons can be activated by novelty in the absence of reward and it is thought that this activation occurs via a polysynaptic pathway that runs from the hippocampus to the dopamine cells of the VTA. However, many aspects of this process remain unclear (see Otmakhova et al., 2013).

Also requiring further investigation are the molecular processes involved in the regulation of protein synthesis related to memory. It is now thought that protein synthesis is not only involved in the consolidation of new memories, but must also be used to re-consolidate memories that have been degraded or "destabilized" as a result of retrieval. In a recent article, Jarome et al. (2016) indicate that CaMKII controls the re-consolidation process through the regulation of proteasome activity. Another mechanism for the regulation of protein synthesis involves MicroRNAs (miRNAs), a class of short, non-coding RNAs. By regulating components of pathways required for learning and memory, miRNAs modulate the influence of epigenetics on cognition in the normal and diseased brain (Saab and Mansuy, 2014).

Another set of important questions relates to the long-standing hypothesis of "Hebbian learning"—the strengthening of synapses between neurons with correlated activity—and its role in memory. At the same time that we are learning more about the mechanisms involved in Hebbian plasticity, we are also learning about how these mechanisms are complemented by synaptogenesis and neuromodulatory processes. Recent research has shown that synaptogenesis is not only important during development, but also plays a central role in associative learning and memory. Synaptogenesis can be triggered by neuron–astrocyte or neuron–neuron contact, and mediated by cell-adhesion proteins including neurexin/neuroligin, Eph receptors, and cadherins, which activate intracellular signaling pathways involving cofilin, GTPases, and other proteins (for a review see Nelson and Alkon, 2015). Others have proposed that Hebbian processes, while important, are not sufficient for memory formation, and must be supported by the activation of neuromodulatory processes, especially in the case of associative aversive learning (Johansen et al., 2014). Another study found evidence for both Hebbian and anti-Hebbian mechanisms of synaptic plasticity, indicating that the mechanisms of learning are highly adaptable (Koch et al., 2013).

Finally, other questions relate to the special role of the amygdala in the emotional enhancement of memory consolidation. It has long been known that emotional arousal contributes to the selection and consolidation of memory. It has also been shown that previously weak or inconsequential information can be strengthened retroactively through an emotional learning experience (Dunsmoor et al., 2015). Evidence suggests that it is the amygdala that is most responsible for the enhancement of memory (de Voogd et al., 2016). As suggested by a review, these developments "will likely lead to an updated view of the amygdala as a critical nexus within large-scale networks supporting different aspects of memory processing for emotionally arousing experiences" (Hermans et al., 2014). Moreover, this research is likely to have important implications for the treatment of psychological disorders (Beckers and Kindt, 2017).

CONCLUSION AND GLOSSARY

There are three main forms of memory: sensory memory, short-term memory, and long-term memory (**Figure 3**). Sensory memory refers to the retention of information coming from the senses. Short-term memory refers to information processed in a short period of time. Working memory performs this processing. Working memory consists of four elements that process information: the central executive (attention control), the visuospatial sketchpad (creates and maintains a visuospatial representation), the phonological buffer (stores and consolidates new words), and the episodic buffer (stores and integrates information from different sources). Long-term memory allows us to store information for long periods of time. This information may be retrieved consciously (explicit memory) or unconsciously (implicit memory). Explicit memory consists of episodic memory (time-related events) and semantic memory (concepts and meanings). Implicit memory has, in turn, procedural memory (motor and executive skills), associative memory (classical and operant conditioning), non-associative memory (sensitization and habituation), and priming (a primary stimulus influencing a secondary one).

Finally, the following glossary includes commentary about the terminology that, in our opinion, is essential for an introductory overview, enabling interested students and professionals to effectively approach the latest memory-related discoveries. This commentary is not intended as an exhaustive definition, but rather collects relevant information to situate the reader within a complex panorama.

Associative memory: refers to the storage and retrieval of information resulting from an association (i.e., resulting from an association with other information). Two types of conditioning are involved in its acquisition: classical conditioning and operant conditioning. Classical conditioning is a kind of associative learning between stimuli and behavior, and operant conditioning is a form of learning in which new behaviors develop in terms of their consequences.

Conceptual short-term memory/episodic buffer: This is a temporary storage system capable of integrating information

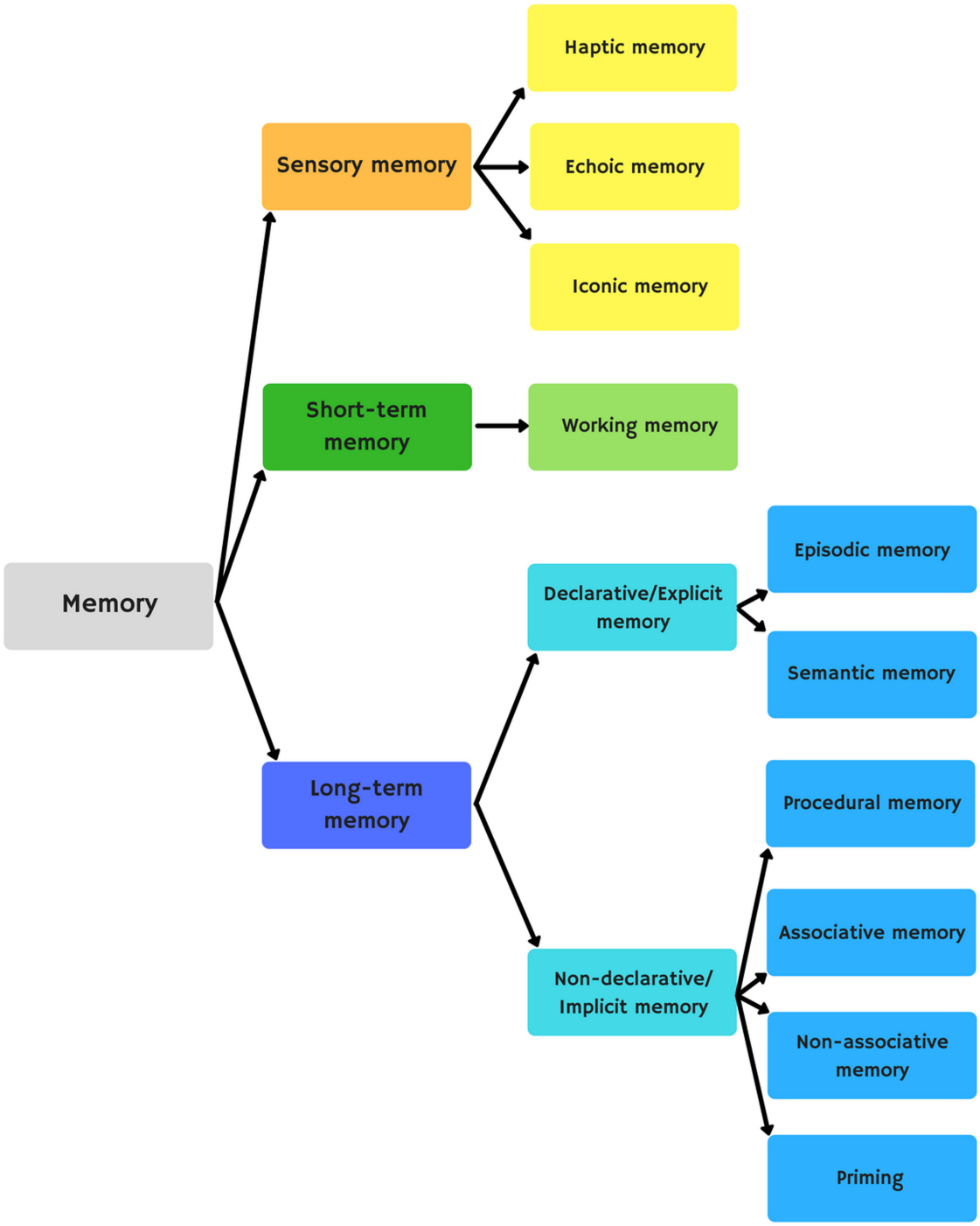

FIGURE 3 | Memory classification.

from different sources that is probably controlled by the central executive. It is episodic in that it has episodes in which information is integrated through space and, potentially, extended through time.

Echoic memory: sensory memory that receives and processes auditory information.

Episodic memory: "involves the ability to learn, store, and retrieve information about unique personal experiences that occur in daily life. These memories typically include information about the time and place of an event, as well as detailed information about the event itself" (Dickerson and Eichenbaum, 2010).

Explicit/declarative memory: refers to conscious memories of previously stored experiences, facts and concepts that are verifiable through a verbal reporting of them (Tulving, 1972).

Haptic memory: sensory memory that receives and processes information from the sense of touch.

Iconic memory: visual-sensory memory that receives and processes visual stimuli.

Implicit/non-declarative memory: this encompasses all unconscious memories, as well as certain abilities or skills. There are four types of implicit memory: procedural, associative, non-associative, and priming memory.

Long-term memory: "refers to the unlimited, continuing memory store that can hold information over lengthy periods of time, even for an entire lifetime. Long-term memory is mainly preconscious and unconscious. Information in long-term memory is to a great extent outside of our awareness, but can be called into working memory to be used when needed. Some of this information is easy to recall, but some is much more difficult to access" (Brodziak et al., 2013).

Non-associative memory: refers to newly learned behavior due to repeated exposure to a single stimulus. The new behavior can be classified into two processes: sensitization and habituation.

Perceptual memory: memory acquired through the senses. It includes a lot of individual experience; it ranges from the simplest forms of sensory memory to the most abstract knowledge.

Priming: an effect whereby exposure to certain stimuli influences the response to subsequently presented stimuli.

Procedural memory: a memory area involved in remembering executive and motor skills necessary to perform a task. It is an executive system that guides activity and usually works on an unconscious level. When necessary, procedural memories are automatically retrieved for use in the implementation of integrated procedures related to motor and intellectual skills.

Semantic memory: refers to the memory of meanings, interpretations and concepts related to facts, information and general knowledge about the world. Semantic memory gives meaning to words and phrases that would otherwise be meaningless and allows for learning based on past experience (Kolb and Whishaw, 2003).

Sensory memory: "Sensory memory is the capacity for briefly retaining the large amounts of information that people encounter daily" (Siegler and Alibali, 2005).

Short-term memory: is the ability to keep a small amount of information available for a short period of time. "Short-term memory should be distinguished from working memory, which refers to structures and processes used for temporarily storing and manipulating information. The relationship between short-term memory and working memory is presented variously by different theories. The notion of working memory is broader and more general because it refers to structures and processes used for temporarily stored and manipulated information" (Brodziak et al., 2013).

Working memory: "The term working memory refers to a brain system that provides temporary storage and manipulation of the information necessary for such complex cognitive tasks as language comprehension, learning and reasoning" (Baddeley, 1992).

Visual memory: constituted by iconic memory, visual short-term and long-term memory.

Visual short-term memory/visuospatial sketchpad: sketchpad's main function is to create and maintain a visuospatial representation that persists through the irregular form found in eye movement and that characterizes our exploration of the visual world (Luck, 2007).

AUTHOR CONTRIBUTIONS

FG drafting of sensory and short-term memory; bibliographical review; introduction and conclusion. Revising critically and final approval of the version to be published. EC drafting of long-term memory (declarative and non-declarative); bibliographical review.

ACKNOWLEDGMENTS

This work was supported by the Institute for Culture and Society (ICS) of the University of Navarra and by the "Programa de Ayudas de la Asociación de Amigos de la Universidad de Navarra." We are grateful to Mind-Brain Group of ICS and, especially, to Nathaniel Barrett Ph.D., Gonzalo Arrondo Ph.D., and Javier Bernácer Ph.D. for their suggestions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 12 May 2017
doi: 10.3389/fphar.2017.00219

Docking Studies and Biological Evaluation of a Potential β-Secretase Inhibitor of 3-Hydroxyhericenone F from *Hericium erinaceus*

Chen Diling[1], Yong Tianqiao[1], Yang Jian[1], Zheng Chaoqun[1,2], Shuai Ou[1] and Xie Yizhen[1]**

[1] State Key Laboratory of Applied Microbiology Southern China, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, Guangdong Open Laboratory of Applied Microbiology, Guangdong Institute of Microbiology, Guangzhou, China, [2] College of Chinese Material Medical, Guangzhou University of Chinese Medicine, Guangzhou, China

OPEN ACCESS

Edited by:
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***Correspondence:**
Chen Diling
diling1983@163.com
Xie Yizhen
xieyizhen@126.com

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 06 January 2017
Accepted: 07 April 2017
Published: 12 May 2017

Citation:
Diling C, Tianqiao Y, Jian Y, Chaoqun Z, Ou S and Yizhen X (2017) Docking Studies and Biological Evaluation of a Potential β-Secretase Inhibitor of 3-Hydroxyhericenone F from Hericium erinaceus. Front. Pharmacol. 8:219. doi: 10.3389/fphar.2017.00219

Alzheimer's disease (AD) is the most common neurodegenerative disorder, affecting approximately more than 5% of the population worldwide over the age 65, annually. The incidence of AD is expected to be higher in the next 10 years. AD patients experience poor prognosis and as a consequence new drugs and therapeutic strategies are required in order to improve the clinical responses and outcomes of AD. The purpose of the present study was to screen a certain number of potential compounds from herbal sources and investigate their corresponding mode of action. In the present study, the learning and memory effects of ethanol:water (8:2) extracts from *Hericium erinaceus* were evaluated on a dementia rat model. The model was established by intraperitoneal injection of 100 mg/kg/d D-galactose in rats. The results indicated that the extracts can significantly ameliorate the learning and memory abilities. Specific active ingredients were screened *in vivo* assays and the results were combined with molecular docking studies. Potential receptor–ligand interactions on the BACE1-inhibitor namely, 3-Hydroxyhericenone F (3HF) were investigated. The isolation of a limited amount of 3HF from the fruit body of *H. erinaceus* by chemical separation was conducted, and the mode of action of this compound was verified in NaN_3-induced PC12 cells. The cell-based assays demonstrated that 3HF can significantly down-regulate the expression of BACE1 ($p < 0.01$), while additional AD intracellular markers namely, p-Tau and $A\beta_{1-42}$ were further down-regulated ($p < 0.05$). The data further indicate that 3HF can ameliorate certain mitochondrial dysfunction conditions by the reversal of the decreasing level of mitochondrial respiratory chain complexes, the calcium ion levels ($[Ca^{2+}]$), the inhibiton in the production of ROS, the increase in the mitochondrial membrane potential and ATP levels, and the regulation of the expression

levels of the genes encoding for the p21, COX I, COX II, PARP1, and NF-κB proteins. The observations suggest the use of *H. erinaceus* in traditional medicine for the treatment of various neurological diseases and render 3HF as a promising naturally occurring chemical constituent for the treatment of AD via the inhibition of the β-secretase enzyme.

Keywords: molecular docking, regulatory mechanism, *Hericium erinaceus*, active pharmaceutical ingredient, functional foods, 3-Hydroxyhericenone F, Alzheimer's disease

INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder, affecting approximately more than 5% of the worldwide population over the age 65, annually. The incidence of the disease is expected to increase in the next 10 years. Despite significant advances in the therapeutic strategies developed for several diseases, AD remains an incurable disease to the majority of the population. AD patients experience poor prognosis and consequently new drugs and therapeutic strategies are required to improve the clinical response and outcome of the patients. AD is a chronic neurodegenerative disease that frequently exhibits a slow progression that is accompanied by a greater recession of the disease over time. The cause of AD is poorly understood, and among various biochemical and morphological events, the presence of neurofibrillary tangles, senile plaques, neuronal and synaptic loss is notably noted (Abbott, 2011). Mitochondrial dysfunction and an accumulation of reactive oxygen species promote redox imbalance and β or tau-induced neurotoxicity (Islam, 2017).

Several studies have documented that mitochondrial dysfunction is related to the aging process and to neurodegenerative disorders (Hirai et al., 2001; Calkins and Reddy, 2011; Swerdlow et al., 2013; Cha et al., 2015). Mitochondria play a central role in the production of ATP as a source of cellular energy via oxidative phosphorylation (Simpkins and Dykens, 2008). In addition, mitochondria exert central roles in apoptosis signaling, lipid synthesis, and intracellular calcium buffering. Mitochondrial functions are less efficient during brain aging and/or brain damage (Cho et al., 2010). Mitochondrial abnormalities have been identified in AD and related neurodegenerative disorders (Reddy, 2011). Aβ has been detected in mitochondria (Thal et al., 2013; Rijal Upadhaya et al., 2014) and has been reported to be transported into the intermembrane space of the mitochondria by the enzyme translocase of the outer mitochondria membrane (Chen and Yan, 2007; Hansson Petersen et al., 2008; Wang et al., 2009). Previous studies demonstrated that Aβ might disrupt mitochondrial function via inhibition of key enzymes in respiratory metabolism, such as α-ketoglutarate dehydrogenase, pyruvate dehydrogenase, and cytochrome oxidase (Casley et al., 2002; Crouch et al., 2005). In addition, the enzyme Aβ-binding alcohol dehydrogenase (ABAD) interacts with Aβ and induces Aβ-mediated toxicity in mitochondria, via the generation of ROS (Lustbader et al., 2004). Furthermore, over-expression of APP and/or exposure to Aβ have been shown to induce mitochondrial fragmentation and abnormal distribution, which result in mitochondrial and neuronal dysfunction (Barsoum et al., 2006; Rui et al., 2006; Wang et al., 2008). Certain studies have suggested dysfunction of mitochondria in Tauopathy patients and disease models (Bratic and Trifunovic, 2010; DuBoff et al., 2012), based on the reduced levels of the mitochondrial metabolic proteins including pyruvate dehydrogenase, ATP synthase, and Complex I (David et al., 2005; Rhein et al., 2009). The mitochondrion is the main source of ROS in the intracellular space, and the over-production of superoxide radical ($O_2^{\bullet -}$) and hydroxyl radical (^{-}OH), due to additional oxidative damage, causes an unrepaired damage to the mitochondrial DNA, thus leading to a functional defect in Complexes I and/or III (Islam, 2017). The aforementioned findings indicate that oxidative stress and mitochondrial dysfunction are associated with AD.

Amnesia is the loss of memory that is caused by hippocampal or medial temporal lobe damage and is a main clinical symptom of AD. During the past years, numerous studies have investigated the molecular mechanisms of learning/memory. As a reducing sugar, D-galactose is able to form advanced glycation end products (AGEs). Administration of D-galactose to human populations can induce cognitive deficits and disruptions in the synaptic communication. Thus, D-galactose-treated rats have been extensively used as a rodent model with synaptic disruption and memory impairment (Huang et al., 2016). The majority of the drugs, including some extracts from traditional Chinese medicines, have been tested for their ability to reverse D-galactose-induced memory deficits (Zhan et al., 2014; Gao et al., 2016; Li et al., 2016).

Sodium azide (NaN_3) is a well-known COX inhibitor, which has been utilized over the last 20 years to induce metabolic compromise, resulting in an increase in amyloid production and the changes in the phosphorylation state of the tau protein (Selvatici et al., 2013; Delgado-Cortés et al., 2015). NaN_3 has been further shown to cause memory deficit and neurodegeneration in rats (Szabados et al., 2004), and is a more potent oxidative stress inducer compared with H_2O_2 (Gao et al., 2007).

Hericium erinaceus belongs to the division of Basidiomycota and the class of Agaricomycetes and is an edible and medicinal mushroom. It is widespread across the continents as a delicacy and replaces pork and/or lamb in the Chinese vegetarian cuisine. *H. erinaceus* is rich in active constituents namely, diterpenoids, steroids, polysaccharides, and other functional ingredients that are used as natural plant resources. *H. erinaceus* exerts a plethora of biological effects namely, cognitive improvement (Mori et al., 2009), stimulation of nerve growth factors (Mori et al., 2008) and nerve cells (Wong et al., 2007) and hypoglycemic and anti-cancer effects (Kim et al., 2014; Li G. et al., 2014). Based

on the aforementioned studies it was assumed that *H. erinaceus* may have potential activity on the treatment of AD, although no reports regarding the pharmacologically active ingredients and their corresponding mode of action have been documented. In the present study, the effects of ethanol:water (8:2) extracts from *H. erinaceus* were investigated on the amelioration of learning and memory processes in a D-galactose-induced deficit rat model. Subsequently, the specific active ingredients were screened by molecular docking studies, in order to identify novel biological components and novel modes of action for the treatment of AD. Furthermore, a NaN_3-induced PC12 cell model was used to confirm the molecular docking studies.

MATERIALS AND METHODS

Learning and Memory Effects Evaluation

D-Galactose-Induced Deficits Rats Prepared and Treatment

Adult male Sprague Dawley rats (180–220 g, obtained from Center of Laboratory Animal of Guangdong Province, SCXK [Yue] 2008-0020, SYXK [Yue] 2008-0085) were pair-housed in plastic cages in a temperature-controlled (25°C) colony room at a 12/12-h light/dark cycle. Food and water were available *ad libitum*. All experimental protocols were approved by the Center of Laboratory Animals of the Guangdong Institute of Microbiology. All efforts were made to minimize the number of animals used.

The rats were randomly divided into four groups as follows: control group that received oral distilled water, model group that received intraperitoneal injection (i.p) of 100 mg/kg/d D-galactose (Zhong et al., 2016; Liang et al., 2017), low-dose group that received i.p D-galactose (100 mg/kg/d), and gavage at a dosage of 50 mg/[kg·d] in ethanol:water (8:2) extracts from *H. erinaceus* (EH), and high-dose group that received D-galactose i.p (100 mg/kg/d) and gavage at a dosage of 100 mg/[kg·d] in ethanol:water (8:2) extracts from *H. erinaceus* (EH) every day in the morning, the dose of EH was according to other literatures (Zhang et al., 2016) proved that *H. erinaceus* extracts at doses of 0.3, 1.0, and 3.0 g/kg for 4 weeks can significantly enhanced the Ach and ChAT concentrations in serum and the hypothalamus in the $AlCl_3$- and D-gal-induced AD mice. Every group consisted of eight animals and the procedure duration was 8 weeks.

Water Maze Tests

Rat spatial learning and memory abilities were tested in the Morris water maze (MWM, DMS-2, Chinese Academy of Medical Sciences Institute of Medicine). The MWM consisted of a circular opaque fiberglass pool (200-cm diameter) filled with water (25 ± 1°C). The pool was surrounded by light blue curtains, and three distal visual cues were fixed on the curtains. A total of four floor light sources of equal power provided uniform illumination to the pool and testing room. A CCD camera was placed above the center of the pool in order to record animal swim paths. The video output was digitized by an EthoVision tracking system (Noldus, Leesburg, VA, USA). The water maze tests included three periods: initial spatial training, spatial reversal training, the probe test and the procedures same to those described previously (Chen et al., 2014).

ADs' Parameters Measurement

The appearance, behavior and the fur color of the animals were observed and documented every day. Animal weight was measured every 3 days during the drug administration period. Following the water maze testing, the blood and serum were acquired. Routine index and cytokines (Wang et al., 2016) were measured and the brains of the animals were dissected. A total of four brains from each group were fixed in 4% paraformaldehyde solution and prepared as paraffin sections. The sections were stained with hematoxylin-eosin (H&E) and immunohistochemistry staining and observed under light microscopy (Zeng et al., 2013; Chen et al., 2014).

Molecular Docking of the Compounds Derived from *H. erinaceus* to the Human β-Secretase (β-site Amyloid Precursor Protein-Cleaving Enzyme 1; BACE1)

The pdb file regarding the crystal structure of human BACE1 with co-crystal ligand (*N*-[(1R)-1- (4- fluorophenyl) ethyl]-*N*′-[(2S,3S)-hydroxy-1-phenyl-4-(1H-pyrazol-1-yl)butan-2-yl]-5-[methyl (methyl sulfonyl)amino] benzene -1,3-dicarboxamide; ZPX394) at the active binding site (3UQU.pdb) was downloaded from the RCSB protein data bank[1] and prepared by deletion of the co-crystal ligand, water, urea, SO_4^{2-} and Cl^{-1} molecules. Subsequently, hydrogen atoms were added to the model and the pH of the protein was adjusted to 7.4. For ligand preparation, the 2D structures of the compounds from *H. erinaceus* were sketched using ChemOffice 2004 and converted into 3D images using the prepare ligands module. The compounds were minimized with Smart Minimizer method using CHARMm force field in DS. The molecular docking procedure was carried out by site-features directed docking (LibDock) for the screen of potent candidates, while the investigation of receptor–ligand interactions was conducted using Discovery Studio 2.5 (DS2.5) according to previously published studies (Panek et al., 2017). The binding site of the protein was defined for all the atoms within 9 Å. The top 10 poses of the docking study were selected on the basis of LibDockScore rank. The interactions between the identified lead compounds and the BACE1 protein were analyzed.

Verification Tests of the Molecular Docking Studies

Preparation of the 3HF

Dried material was loaded onto a column that was eluted with twofold greater volume of petroleum ether:ethanol (9:1, PE) for the volatile compounds and the fatty acid fraction. The samples were sequentially eluted with fourfold greater volume of ethanol:water (8:2) for the crude extraction and the elute was dried and loaded on the D-101 macroporous resin. The samples were subsequently eluted with twofold greater volume of petroleum ether and fourfold greater volume of acetone

[1]http://www.pdb.org

consecutively. The acetone elute was collected and evaporated to dryness. The extraction and separation process was developed in-house and the patent application is pending, and the extraction steps showed in **Figure 5A**.

Cell Culture and Treatment

PC12 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). They were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and seeded in 25 cm^2 culture dishes (Chen et al., 2013). The cells were initially stabilized at 37°C for 24 h, at 80% of confluence, and subsequently cultured in serum free medium following passaging. The cells were incubated with different concentrations of 3HF (final concentrations: 0.05, 1.00, and 1.50 μg/ml) for 2 h. NaN_3 was incubated at a final concentration of 0.03 mM to the cell culture for an additional 24 h. All the cells used in the present study were undifferentiated.

Cell Viability Assay

The cell viability of PC12 following treatment of 3HF was measured by quantitative colorimetric assay using the cell counting kit-8 (CCK-8, Dojindo Laboratories, Japan) assay. The assay was conducted as described previously (Xiong et al., 2015). The cells were seeded in 96-well culture plates at a density of 2×10^4 cells/well. Following drug treatment, 10 μL/well of CCK8 solution was added, and the cells were incubated at 37°C for 1 h. The optical density of each well was determined at 450 nm using a microplate reader (RT-2100C, USA). Cell viability was expressed as percentage of survival compared with the non-treated control. The data are presented as the mean and standard error of the mean (SEM) for three independent experiments.

Measurement of Intracellular ROS Production, Mitochondrial Membrane Potential, and $[Ca^{2+}]_i$

The intracellular ROS level was measured using the 2′, 7′-dichlorofluorescein diacetate (DCFH-DA) method (Chen et al., 2013), whereas the mitochondrial membrane potential was measured using rhodamine 123 fluorescent dye (Chen et al., 2013). The calcium ion concentration $[Ca^{2+}]_i$ was measured using the Fura-2/AM fluorescent dye (Chen et al., 2013). The results are expressed as percentage of activity compared with the non-treated control.

Measurement of Intracellular F_1F_0-ATPase, ATP, and NADH-CoQ Levels

The cells were seeded in 25 cm^2 culture dishes. At 80% confluence, the cells incubated with different concentrations of 3HF (final concentrations: 0.05, 1.00, and 1.50 μg/ml) for 2 h. NaN_3 was used at a final concentration of 0.03 mM and was subsequently added to the culture for an additional 24 h. At the end of the drug treatment, the cells were washed with D-Hanks solution, scraped from the plates in the presence of 1 mL ice-cold PBS (0.1 M, containing 0.05 mM EDTA) and homogenized. The homogenate was centrifuged at 4,000 × *g* for 10 min at 4°C. The resulting supernatants were stored at −80°C until further analyses. The activity of the enzymes F_1F_0-ATPase, NADH-CoQ reductase and the ATP levels within the mitochondria was determined using corresponding kits as demonstrated previously (McKenzie et al., 2007). The protein concentration of the cells was determined using the Coomassie Brilliant Blue G250 assay. The enzyme activities and protein content were all determined using the Detection kits purchased from Nanjing Jiancheng Bioengineering Insititute (Nanjing, Jiangsu, China). The procedures were carried out according to the manufacturer instruction. The levels were normalized to the protein concentration of each sample and expressed as a percentage of activity compared with the non-treated control.

Cell Mitochondrial Morphology

The presence of viable mitochondria was identified by Mito-tracker green (Molecular Probes, Beyotime, Shanghai, China) staining as described by Chang et al. (2014) with minor modifications. The stock solution of Mito-tracker green was prepared at a concentration of 1 mM in DMSO and stored at −20°C. The cells were stained in PBS with Mito-tracker Green (0.2 mM) at 37°C for 10 min. Following washing of the samples with PBS, the embryos were visualized using a fluorescent microscope at 490 nm.

Transmission Electron Microscopy (TEM)

The cells were fixed in 2.5% glutaraldehyde in PBS (pH 7.3). Following treatment with 1% osmium tetroxide solution (OsO_4), 2% uranylacetate (UA) and dehydration in ethanol and acetone solvents, the samples were embedded in epoxy resin and polymerized for 48 h at 60°C. Ultrathin sections were cut using an ultramicrotome and placed on copper net (150 mesh). The sections on the grids were post-stained for 2 min with 1% UA, and for 6 min with 1% lead citrate by the addition of single drops of the staining solution at room temperature. The sections were rinsed in deionized water, dried and finally observed using a H7650 electron microscope (Hitachi, Tokyo, Japan), as described in previous studies (Ghobeh et al., 2014).

COX I, COX II, NF-κB, Caspase-3, and Caspase-9 Assays

Following drug treatment, the cells were washed with D-Hanks solution, scraped from the plates in the presence of 1 mL ice-cold PBS (0.1 M, containing 0.05 mM EDTA) and homogenized. The homogenate was centrifuged at 4,000 × *g* for 10 min at 4°C. The resulting supernatants were kept at −80°C until further analyses. The activities of COX I, COX II, NF-κB, Caspase-3, and Caspase-9 enzymes were measured by the Detection kits purchased from Cloud-Clone, Corp. (Houston, TX, USA). The procedures were carried out according to the manufacturer instruction. The levels of enzyme activity were normalized according to the protein concentration of each sample and expressed as a percentage of activity compared with non-treated control.

Preparation of Total RNA and Quantitative Reverse Transcriptase PCR (Q-RT PCR)

Cells were seeded in 25 mL plastic flask at a density of 2×10^5 cells/mL and incubated with different concentrations of 3HF (final concentrations: 0.05, 1.00, and 1.50 μg/ml, respectively) for

2 h. NaN_3 was used at a final concentration of 0.03 mM and was added to the culture for an additional 4, 8, or 24 h. The cells were harvested and the total RNA was extracted using Trizol reagent, whereas the remaining DNA was removed by DNase I. Consequently, purified RNA was obtained and the yield and purity was calculated by spectrophotometric estimation of the OD value at 260 and 280 nm respectively (k2800, Beijing Kai'ao Company).

A q-RT PCR assay was conducted using RNA obtained from PC12 cells. The expression levels of p21, PARP1, and NF-κB in mitochondria were examined. Amplification was carried out using ABI ViiA 7 Detection System and Hema9600 PCR (Zhuhai Heima medical equipment corporation) instruments. The mRNA levels of a control group were used as the baseline; As a result, $^{\Delta\Delta}$Ct was calculated using the formula $^{\Delta\Delta}C_T = {}^{\Delta}C_T$ of target gene $-\Delta C_T$ of the baseline. The fold change of the mRNA levels was calculated as fold $= 2^{-\Delta\Delta CT}$. The PCR primer sequences were as follows: sense: TCTTCTGCTGTGGGT CAGGAGG and antisense: GGCAGGCAGCGTATATCAGGAGA for p21;sense: TGCTGTCAAGGAGGA AGGTGTC and antisense: TCCAGGA CGTGTGCAGAGTGTT for PARP1; sense: TGAGGAAGAGGC ATG TAGAGACT and antisense: ACTGGCACTTCGGACA ACAGAAG for NF-κB.

Western Blotting Analysis

Cells were seeded in 6-well culture plates at 5×10^6 cells/well and were washed twice with D-Hanks solution following drug treatment. The cells were harvested and lysed with protein lysis buffer and the concentration of the protein was determined using the Coomassie Brilliant Blue G250 assay kit (Nanjing JianCheng Bioengineering Institute, China). Protein samples were separated by SDS-PAGE electrophoresis for 1.5 h at 90 V. The separated proteins were transferred to PVDF membranes using a transblotting apparatus (Bio-Rad Laboratories, USA) for 90 min at 90 V. The membranes were blocked with 5% (w/v) non-fat milk in TBS-T (Tris-buffer saline containing 0.1% Tween-20) at room temperature for 30 min and subsequently incubated at 4°C overnight with appropriate amount of primary antibody against BACE1 (ab183612), p-Tau (ab64193), $A\beta_{1-42}$ (ab201060), PARP1 (ab191217), p21 (ab80633), and NF-κB p65 (ab16502) proteins. The primary antibodies were purchased from Abcam, Cambridge, UK, with the exception of the antibody for ARP1 (sc-393500) that was purchased from Santa Cruz Biotechnology, Inc. Following binding with the primary antibody, the membrane was washed with TBS-T once for 15 min and subsequently for 5 min for three times. The membrane was probed with horseradish peroxidase-conjugated secondary antibody at 4°C overnight. Incubation of the membranes with primary antibody against GAPDH at 4°C overnight, followed by a horseradish peroxidase-conjugated goat anti-mouse IgG at 37°C for 2 h was used as a loading control. The membrane were further washed with TBS-T for three times and the protein bands were visualized by ECL western blotting detection reagents (Amersham Biosciences, Buckinghamshire, UK).

Statistical Analysis

Data are expressed as mean ± SD. Multiple group comparisons were conducted using one-way and two-way mixed analysis of variance (ANOVA) followed by Dunnett's test in order to detect inter-group differences. A difference was considered statistically significant if the p-value was less than 0.05. Statistical Package for the Social Science (SPSS 17.0, SPSS Inc.) was used for statistical analysis in this study.

RESULTS

Effects on Learning and Memory

Animal Appearance and Weight Control

The fur of the EH-treated animals was apparently smoother compared with that of the model group, which indicated that treatment with the EH can retain the fur at a healthy state. The weight records indicated that there were no significant difference ($F = 2.55$, $p > 0.05$) in the average weight change rate between the treated and the model groups, as shown in **Figure 1A**. The mean weight of the animals was approximately 330 ± 11.27 g at the beginning and 475 ± 20.00 g at the end of the experiment, which implied that the drugs exhibited no serious adverse effects on the animal weight.

Effects on Behavior

The incubation period for each EH-treated group (62.85 ± 11.97 s) was significantly shorter in length compared with that noted in the model group. The low-dose EH group incubation period was 74.16 ± 15.61 s, while the incubation period for the high-dose EH group was 66.73 ± 10.56 s on the first day. The differences noted in the incubation periods of the aforementioned groups were significant ($F = 9.66$, $p < 0.05$) compared with those noted in the model group (incubation period of 94.25 ± 12.20 s). On the fifth day, the low-dose EH group incubation period was 24.94 ± 5.30 s, whereas for the high-dose group the corresponding value was 21.98 ± 4.71 s. The differences were significant compared with the model group 67.65 ± 9.71 s ($F = 98.71$, $p < 0.01$), as shown in **Figure 1B**. Furthermore the swimming time in NW quadrant was improved (**Figure 1C**). The results indicated that EH can ameliorate D-galactose-induced learning and memory dysfunction in rats.

The probe test results indicated that there were no significant differences ($p > 0.05$) among the groups with regard to the total swimming distance and/or speed. The swimming time of the control group in the NW quadrant (33.12 ± 12.31 s) was longer compared with that noted in the other three quadrants, and the differences among them were significant ($F = 6.63$, $p < 0.01$). The swimming time of the model group was 17.23 ± 7.43 s, which was significantly lower compared with the control group ($p < 0.01$), suggesting that the rats couldn't remember the location of the platform. The swimming times of the low- and high-dose EH groups were 31.55 ± 8.87 s, 36.66 ± 9.26 s in the NW quadrant, which were significantly greater in length compared with the model group ($F = 6.63$, $p < 0.01$). The differences noted in the parameters measured of the aforementioned groups were significant ($F = 6.63$, $p < 0.01$), as shown in **Figure 1D** compared with those noted in the model group. In addition, the swimming trajectory of the EH group was apparently denser in the NW quadrant compared with the other quadrants, as shown

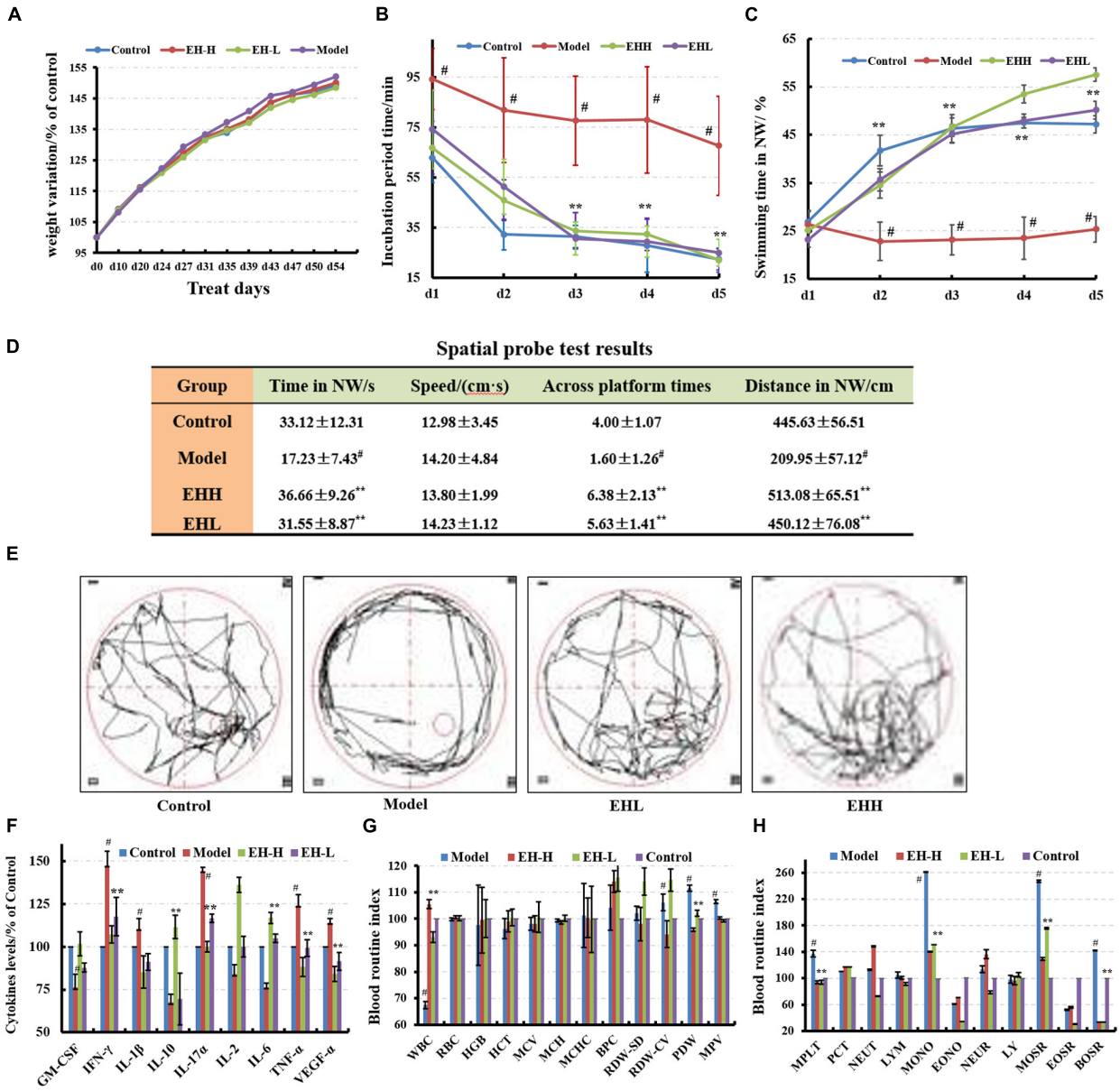


FIGURE 1 | Effects of ethanol:water (8:2) extracts from *Hericium erinaceus* (EH) on D-galactose-induced deficit in rats. (A) Body weight changes during the treatment; **(B,C)** water maze tests results at the specified incubation periods and swimming times in NW; **(D)** spatial probe test results; **(E)** swimming trajectory during the spatial probe test; **(F)** cytokine levels of GM-CSF, TNF-γ, 1L-10, IL-2, 1L-17α, 1L-6, TNF-α, and VGEF-α in serum; **(G,H)** routine blood index changes. Control group (oral distilled water), model group [intraperitoneal injection (i.p) of 100 mg/kg/d D-galactose], low-dose group (concomitant administration by i.p injection of 100 mg/kg/d D-galactose and gavage at a dose of 50 mg/[kg·d] ethanol:water (8:2) extracts from *H. erinaceus* (EH)), high-dose group (concomitant administration by i.p injection of 100 mg/kg/d D-galactose and gavage at a dose of 50 mg/[kg·d] ethanol:water (8:2) extracts from *H. erinaceus* (EH)). Values are expressed as mean ± SD, $^{\#}p < 0.05$ vs. control group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. model group, indicates significant differences compared with the model group.

in **Figure 1E**. The results suggested that EH could ameliorate D-galactose-induced learning and memory dysfunction in rats.

Improvement on AD Parameters

Cytokines levels and blood routine index changes

The cytokine levels in the serum of the model group were significantly different from those noted in the control group ($p < 0.05$), and the levels of the pro-inflammatory cytokines increased, while the corresponding levels of the anti-inflammatory cytokines decreased (**Figure 1F**). Following treatment by EH, the cytokine levels were reversed to the levels noted in the control group, which indicated that treatment with the EH improved the inflammatory environmental factors. Certain blood biomarkers of the model group were apparently changed compared with the control group, while following treatment of EH, their levels were reversed to the levels noted in

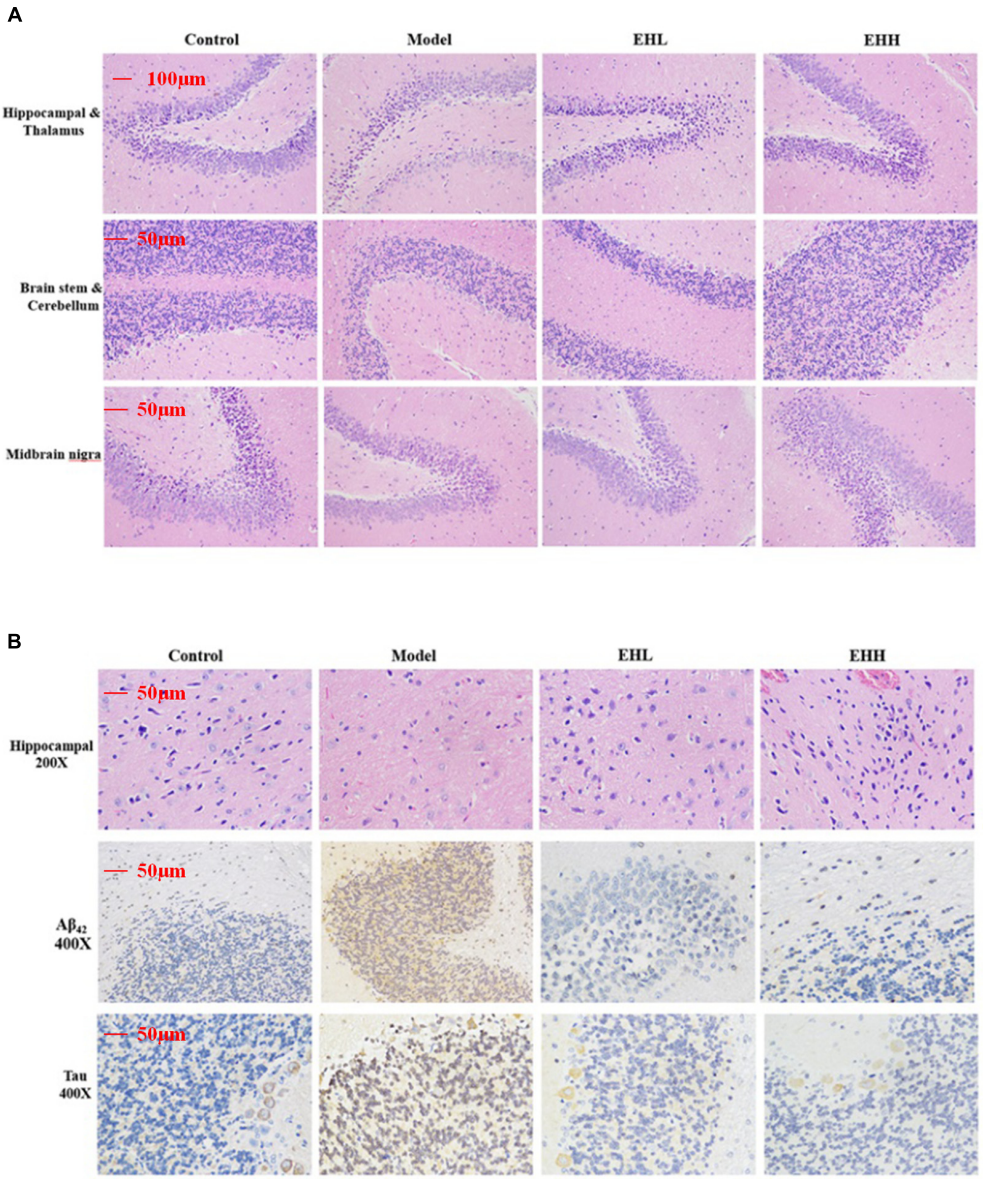


FIGURE 2 | Histopathological changes and immunohistochemistry staining of Tau and Aβ$_{42}$ in the brain tissues of D-galactose- induced deficit rats. (A) is the hematoxylin-eosin (HE) staining of hippocampal and thalamus, brain stem and cerebellum, midbrain nigra; **(B)** is HE staining of hippocampal, immunohistochemistry staining of the expression of Aβ42 and Tau protein in hippocampal.

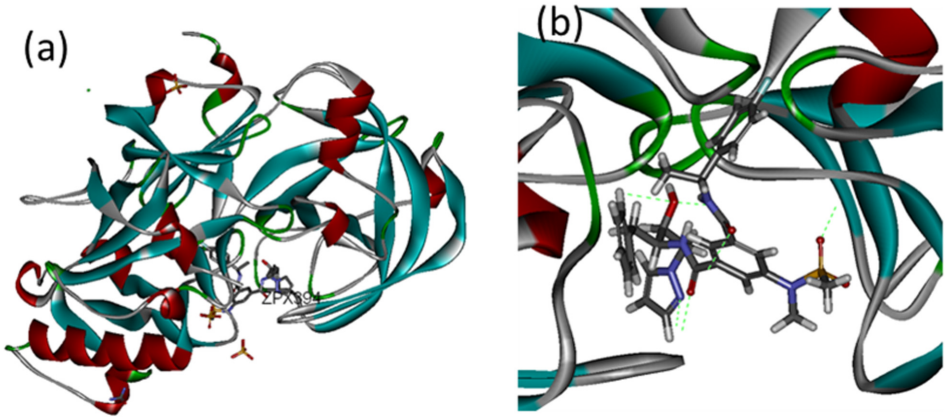
the control group (**Figures 1G,H**). This suggested that the EH can retain the D-galactose-induced deficit in the rat model.

Pathological and morphological findings

Following hematoxylin-eosin (HE) staining, the histopathologic morphology of the model group exhibited apparent changes compared with the control group (**Figure 2A**), which indicated that the size of neurons in the cortex was smaller, and the cells were associated with nuclear pyknosis. The boundary of the cytoplasm and the nucleus was not distinct (**Figure 2B**). The cellular space between the neurons and the neurogliocytes had expanded, whereas the hippocampal pyramidal neuron had become smaller (**Figure 2B**). Following treatment with EH, the pathological changes noted in the model group were improved and the effect was proportional to the dose of administration.

Immunohistochemistry staining indicated that the expression of Tau (3.21 ± 0.17 fold of control) and Aβ$_{42}$ (4.67 ± 0.28 fold of control) proteins in the brain tissues of D-galactose-induced rats was significantly increased compared with the control group (as onefold), while following treatment with EH, the changes noted were attenuated, especially the high dose group (0.69 ± 0.12 for Tau, 0.94 ± 0.11 for Aβ$_{42}$). The pharmacological effect caused by EH treatment was proportional to the dose of administration.

A



B

Docking score of compounds from Hericium erinaceus and co-crystal ligand (ZPX394)

Compounds	LibDockScore
ZPX394	211
15	180
7	176
6	169
12	164
10	158

C

Intra and intermolecular hydrogen bonds

Donor-Hydrogen…Acceptor	DH (Å)	H…A (Å)	D-H…A (°)	HAY (°)
THR72:HN - 15:O41	1.00	2.06	159.5	117.017
LYS321:HZ1 - 15:O42	1.04	2.06	109.3	93.3352
LYS321:HZ2 - 15:O21	1.04	2.29	109.02	110.46
LYS321:HZ3 - 15:O42	1.04	2.44	86.81	83.2867

D

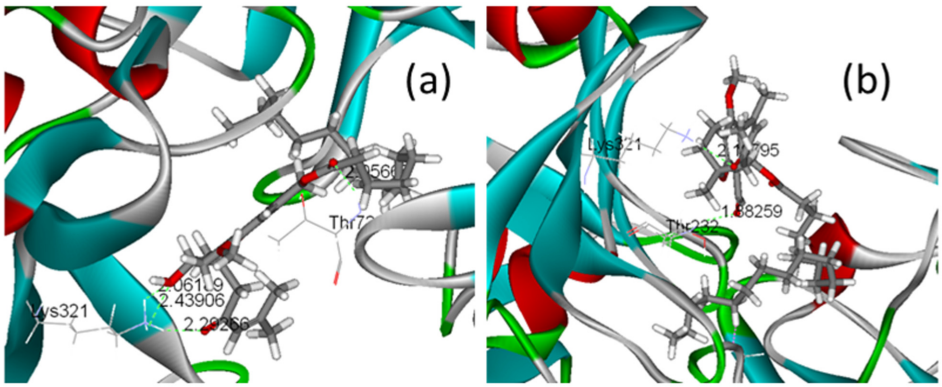


FIGURE 3 | Binding pattern of *H. erinaceus* compounds: (A-a) crystal structure of BACE1; **(A-b)** binding modes and co-crystal ligand in BACE1; **(B)** docking scores of compounds from *H. erinaceus* and co-crystal ligand (ZPX394); **(C)** intra and intermolecular hydrogen bonds; **(D-a)** crystal structure of BACE1 and **(D-b)** 3-Hydroxyhericenone F-mediated hydrogen bond interactions with Lys321 and Thr72 (green line).

Molecular Docking of Compounds Extracted from *H. erinaceus* to BACE1 Enzyme and Investigation of the Receptor–Ligand Interactions

The molecular docking study was conducted using the LibDock protocol for the screen of potent candidates from *H. erinaceus* and the investigation of receptor–ligand interactions in the Discovery Studio 2.5 (DS2.5) software. A total of 17 components from *H. erinaceus* were collected from a literature search and 1048 poses were generated for all the compounds investigated. The docked poses were ranked by the LibDockScore and the top 10 poses with the co-crystal ligand for BACE1 were retained (**Figure 3B**). The data revealed four hits namely, compounds 15 (3HF), 7, 6, and 12 with corresponding scores of 180, 177, 169, and 164, respectively compared with the score noted for ZPX394 (211) (**Figure 3B**). This indicated that the four compounds identified may exert potent BACE1 inhibitory activity. The interaction between the BACE1 protein and the four compounds identified was further analyzed using

Receptor–Ligand Interaction module in DS. The analysis between BACE1 and compound 15 (3HF) revealed that four hydrogen bond interactions appeared in the docked pose that were the following: the oxygen of the hydroxyl group was attached to the third position of the *O*-substituted hexyl ring, whereas the oxygen of the carbonyl group was attached on the second position of the 3-ene-4-methyl-2-pentone moiety of compound 15. This facilitated the interaction with the amine group of LYS321 with interference distance values of 2.062, 2.293, and 2.439 Å, respectively. The oxygen of the carbonyl group between the long alkyl group and the phenyl ring interacted with the amine group of Thr72 with interference distance value of 2.056 Å (**Figure 3C**). The interaction with LYS321 forms two 3-membered and 9-membered rings, which may stabilize the conformation. Furthermore, there is no bump between compound 15 and BACE1. This suggested optimal interaction between compound 15 and BACE1. Since compound 7 exhibited a high LibDock score (176), we further analyzed the interactions between compound 7 and the BACE1 protein. The oxygen of the carbonyl group was attached on the second position of the 3-ene-4-methyl-2-pentone moiety of the compound 7. This allowed an interaction with the amine group of LYS321 with interference distance value of 2.198 Å. Finally, an interaction of the amine group of Thr232 was observed with the oxygen atom of the carbonyl group attached on the phenyl ring of compound 7, with interference distance value of 1.883 (**Figure 3**).

Verification of the Molecular Docking Studies

3HF Significantly Inhibits the Expression of BACE1 in NaN_3-Induced PC12 Cells

The phosphorylation status of the intracellular markers that are involved in the neurodegenerative processes, such as tau, $A\beta_{1-42}$ and beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) was assessed by western immunoblotting. The expression of the modulators BACE1, p-Tau and $A\beta_{42}$ was investigated in NaN_3-induced PC12 cells. The cells were pretreated with 3HF at the concentrations of 0.05, 1.00, and 1.50 μg/ml for 2 h, followed by exposure to 0.03 mM of NaN_3 for 4, 8, and 24 h (**Figure 4**). The expression of BACE1, p-Tau and $A\beta_{42}$ proteins were decreased in all three 3HF treatment groups compared with the normal group. The expression of the proteins p21, NF-κB p65, and PARP1 that are related to mitochondrial dysfunction or cellular aging was further investigated. The data suggested that the expression levels of the aforementioned proteins were reversed following treatment with EH compared with the model group.

Effects of 3HF on the mRNA Expression Levels of Six Genes Related to Mitochondrial Dysfunction

The mRNA expression of six genes that are considered to be associated with the mitochondrial dysfunction processes was investigated in order to add further insight in the mechanism involved in the improvement of the mitochondrial dysfunction caused by 3HF (Picone et al., 2014; Ajith and Padmajanair, 2015). The cells were pretreated with 3HF at the concentrations of 0.5, 1.00, and 1.50 μg/ml for 2 h and further exposed to 0.03 mM of NaN_3 for 4, and 8 h. The mRNA expression levels of p21, NF-κB p65, and PARP1 in the mitochondria were measured. The PARP1, p21, and NF-κB p65 expression levels increased following treatment from 4 to 8 h with 0.03 mM of NaN_3. These increased trends were reversed by co-administration with different concentrations of 3HF, notably at the high 3HF dose (1.50 μg/ml) group, while the mRNA expression of the aforementioned genes was reversed to normal levels (**Figure 4**).

3HF Improves the Cell Viability of NaN_3-Induced PC12 Cells

Cell viability was assessed by the CCK-8 assay. PC12 cells were treated with different concentrations of 3HF for 24 h, and the cell viability was compared between 3HF-treated groups and control groups, showed in **Figure 5B**. When the cells were treated with 3HF at a concentration range of 0.01 to 2.00 μg/ml for 24 h, the cell viability was significantly increased compared with the control group (100%), while at the concentration of 5.00 μg/ml, the cell viability was decreased compared with the 2.00 μg/ml-treated group but increased compared with the control group. The results indicated that 3HF under 5.00 μg/ml was considerably non-toxic in PC12 cells.

Treatment of PC12 cells with a concentration range of 0 to 0.1 mM of NaN_3 for 24 h induced cytotoxicity (**Figure 5C**). The cell viability was reduced to 24.74% of the control value (100%), as shown in **Figure 5B**. When the cells were co-incubated with 3HF at the concentrations of 0.05, 1.00, and 2.00 μg/ml for 2 h and further exposed to 0.03 mM of NaN_3 for 24 h, the cell viability was significantly increased (63, 85, and 106% of the control value, respectively) compared with the NaN_3 group (**Figure 5D**). The results indicated that 3HF conferred a protection against the NaN_3-induced cytotoxicity in PC12 cells.

Effect of 3HF on NaN_3-Induced Intracellular ROS, $[Ca^{2+}]_i$ and Mitochondrial Membrane Potential in PC12 Cells

Treatment of PC12 cells with 0.03 mM of NaN_3 for 24 h caused a significant increase in the Calcium ion $[Ca^{2+}]_i$ levels (**Figure 5F**, 189.31% of the control value) with a concomitant decrease in the mitochondrial membrane potential (**Figure 5G**, 46.31% of the control value). When the cells were pretreated with 3HF at the concentrations of 0.05, 1.00, and 1.50 μg/ml for 2 h and further exposed to 0.03 mM of NaN_3 for 24 h, the $[Ca^{2+}]_i$ levels were significantly reduced to 164.84, 130.26, and 103.16%, respectively, of the control value, while the mitochondrial membrane potential of the PC12 cells was significantly increased (66.84, 85.26, and 93.17% of the control value, respectively) compared with the NaN_3 group. In addition, the ROS levels indicated a similar pattern of change with that noted in the $[Ca^{2+}]_i$ levels (**Figure 5E**).

Influence of 3HF on the Activities of ATP, NADH-CoQ, and $F_{(1)}F_{(0)}$-ATPase in the Mitochondria of the PC12 Cells

The mitochondria complexes I, V and ATP levels were markedly increased in the 1.00 and 1.50 μg/ml 3HF treated-groups, while

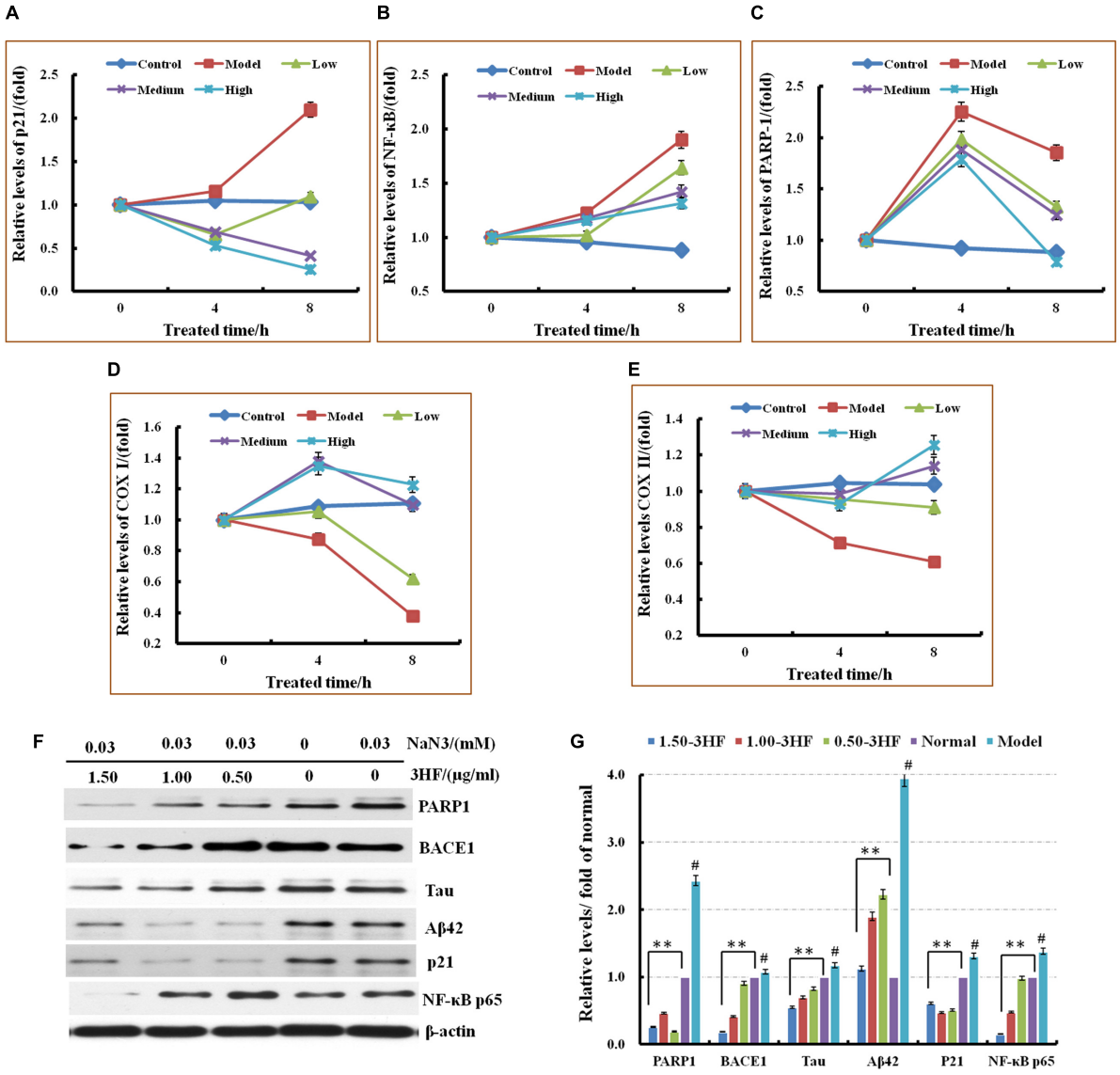


FIGURE 4 | Effects of 3HF on the mRNA expression levels of PARP1, p21, NF-κB p65, COX I, and COX II (A–E) and the expression of the proteins related to mitochondrial dysfunction. 3HF reduced BACE1 although it reduced p-Tau and $A\beta_{42}$ expression **(F,G)**. Cells were pretreated with 3HF at the concentrations of 0.05, 1.00, and 1.50 μg/ml for 2 h, followed by exposure to 0.03 mM of NaN_3 for 12 h, and the protein extracts were used for Western blot analysis of the indicated proteins. The blots were probed with β-actin for the loading control validation $^{\#}p < 0.01$ compared with control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the NaN_3-treated group.

the latter treatment of 3HF slightly increased complex I activity and ATP levels compared with the model groups (**Figures 5H–J**).

Effects of 3HF on the Expression of COX I, COX II, NF-κB, Caspase-3, and Caspase-9 Proteins

The activities of COX I, COX II, NF-κB, Caspase-3, and Caspase-9 proteins were detected using an ELISA method in order to clarify the protective mechanism of 3HF on the NaN_3-induced PC12 cells. The levels of COX I (**Figure 5K**) and COX II (**Figure 5L**) were significantly reduced when the cells were treated with 0.03 mM NaN_3 for 24 h, while the NF-κB (**Figure 5M**), caspase-3 (**Figure 5N**), and caspase-9 (**Figure 5O**) levels were significantly reduced. Following pretreatment of the cells with 3HF at the concentrations of 0.05, 1.00, and 1.50 μg/ml for 2 h and subsequent exposure to 0.03 mM of NaN_3, the protein (COX I, COX II, NF-κB, Caspase-3, and Caspase-9) expression was approximately reversed to the normal levels.

Cell Mitochondria Morphology

The morphology of the mitochondria was evaluated by microscopy (phase-contrast, fluorescence, and electron microscopy) following treatment of PC12 cells by 0.03 mM of NaN_3. The morphological assessment revealed swelling, shrinking cells and lower fluorescence intensity (38.19% of

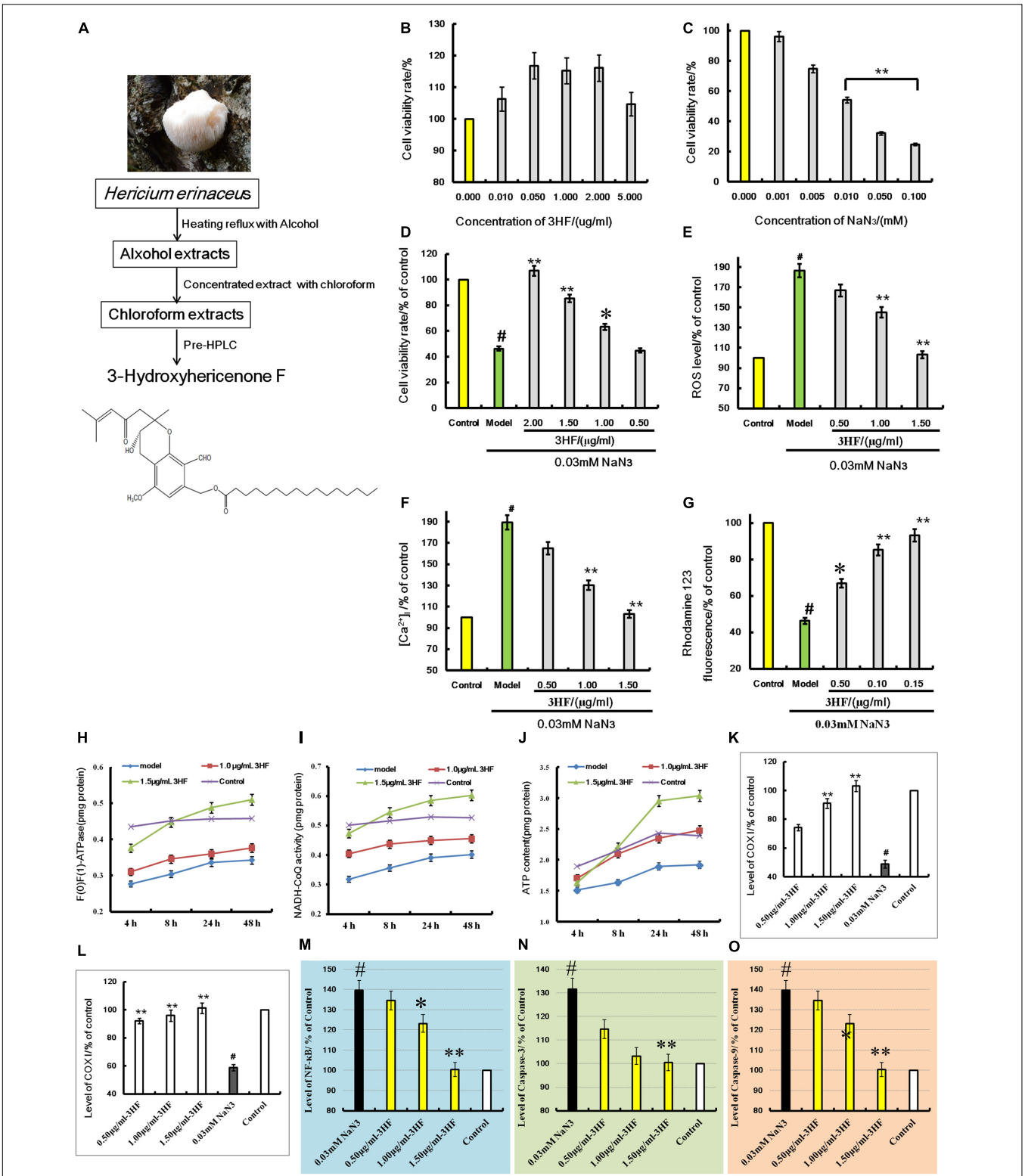


FIGURE 5 | (A) is the extraction flowchart and chemical structure of 3HF. Effects of 3HF and NaN_3 on PC12 cells **(B–D)** and effect of 3HF on ROS **(E)**, $[Ca^{2+}]_i$ **(F)**, mitochondrial membrane potential **(G)**, $F_{(1)}F_{(0)}$-ATPase **(H)**, NADH-CoQ **(I,J)**, COX I **(K)**, COX II **(L)**, NF-κB **(M)**, Caspase-3 **(N)**, and Caspase-9 **(O)** in NaN_3-induced cytotoxicity PC12 cells. The cells were pretreated with 3HF at the concentrations of 0.05, 1.00 and 1.50 μg/ml for 2 h, followed by exposure to 0.03 mM of NaN_3 for 24 h, whereas the control group was treated with PBS. Values are provided as the mean ± SD (n = 5) and % of the control group, $^{\#}p < 0.01$ compared with control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the NaN_3-treated group, indicates significant differences compared with the model group.

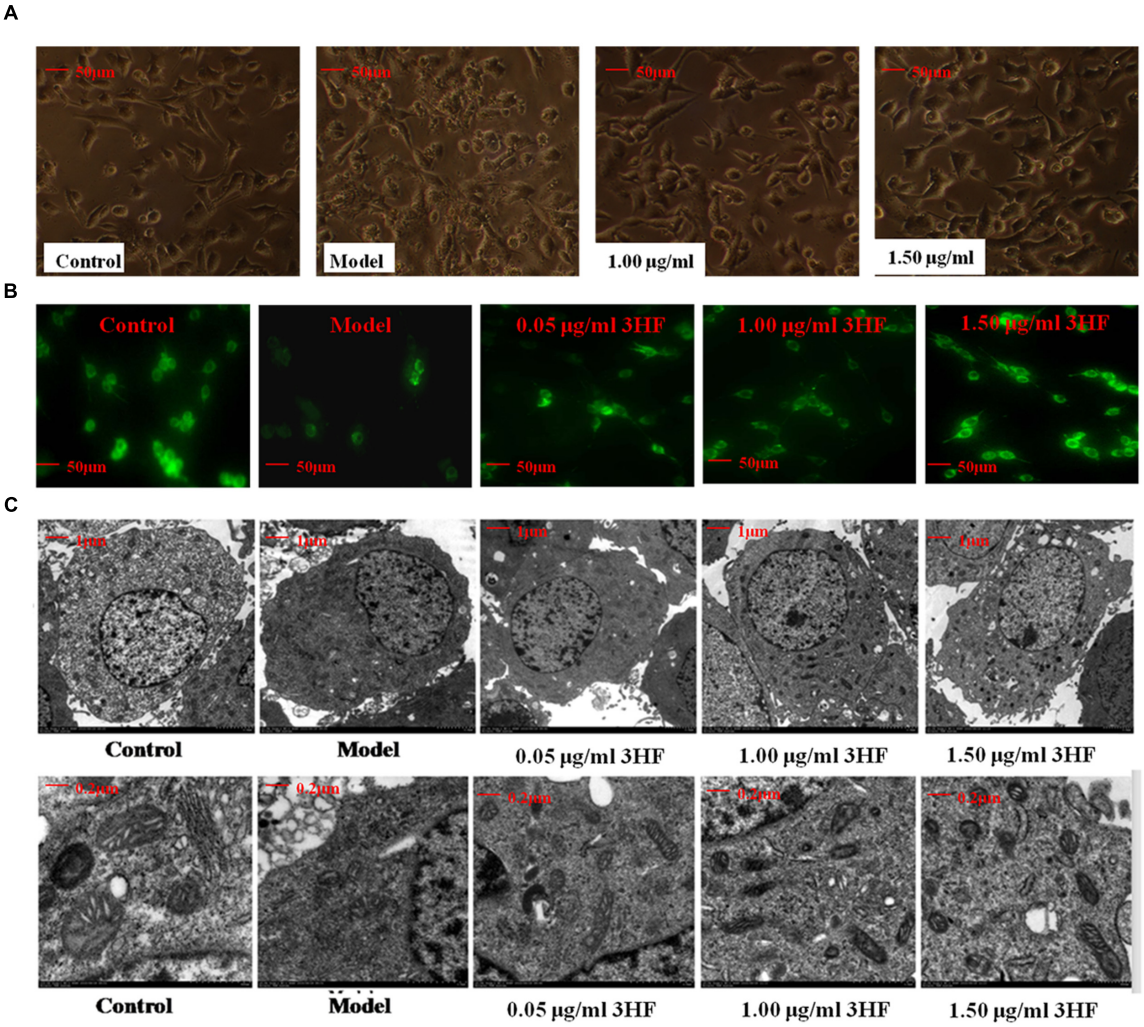


FIGURE 6 | Effect of 3HF on the cellular and mitochondrial morphology. (A) The investigation was conducted by inverted microscope; **(B)** The investigation was conducted by fluorescent microscope at the wavelength of 490 nm. The number of cells and fluorescence intensity of the model group (treated with 0.03 mM NaN_3 for 24 h) were lower compared with the control, while the 3HF increased the number of cells and fluorescence intensity; **(C)** The analysis was carried out by electron microscopy (1 and 5K). The 0.03 mM NaN_3 treated groups indicated an ultrastructural change with disappearance of the nucleolus, condensed chromatin that was localized to the inner side of an intact nuclear membrane, blur mitochondria with disintegration and substantial lysis of cristae in PC12 cells.

control) compared with the control samples, while in the 3HF-treated groups these effects were attenuated (**Figures 6A,B**), the fluorescence intensities were increased to 43.15, 61.27, and 86.78% of the control value, respectively. Furthermore, we used electron microscopy to determine the ultrastructural changes in the cells following NaN_3 treatment. The data indicated that the nucleolus disappeared and the condensed chromatin was localized to the inner side of an intact nuclear membrane (**Figure 6C**). The mitochondria exhibited blur appearance and the boundary was not clear with concomitant disintegration and lysis of the cristae in the NaN_3-treated PC12 cells compared with untreated control cells (**Figure 6C**). Based on the aforementioned observation, it was concluded that 3HF significantly alleviated the NaN_3-induced ultrastructural changes that were characteristic of cellular damage (**Figure 6C**).

DISCUSSION

Various food constituents have been proposed as disease-modifying agents for AD, due to epidemiological evidence of their beneficial effects, and due to their ability to ameliorate the factors that are associated with the pathogenesis of AD pathogenesis (Chan et al., 2016). Such constituents have been demonstrated to bind iron, copper and zinc, scavenge reactive oxygen species and suppress the fibrillation of amyloid-beta peptide (Aβ), thus contributing to the prevention of AD (Chan et al., 2016). Mushrooms were originally used as a nutritional supplement, although in certain cultures, they have been traditionally exploited for their potential as medicinal remedies for various diseases. A majority of medicinal mushrooms were reported to affect the nervous

system namely, *Lion's Mane*, *H. erinaceus* and *Tiger Milk*, *Lignosus rhinocerotis*. The latter two mushrooms can stimulate neurite outgrowth (Samberkar et al., 2015), whereas *Phellinus igniarius* can reduce transient cerebral ischemia-induced neuronal death, suppress oxidative injury and disrupt the blood–brain barrier via microglia activation (Kim et al., 2015). *Agaricus blazei* protects the brain against oxidative stress-induced damage and increases mitochondrial-coupled respiration (de Sá-Nakanishi et al., 2014). *Hericium ramosum* mycelia extracts exert antioxidant activity and NGF synthetic activity. It has been shown that Dictyoquinazols extracted from *Dictyophora indusiata* protect primary cultured mouse cortical neurons from glutamate-and NMDA-induced excitotoxicities (Lee et al., 2002). Furthermore, the methanolic extract of *Chaga* enhances the cognitive and anti-oxidant activities of scopolamine-induced experimental amnesia (Giridharan et al., 2011). In addition, the oligosaccharide fraction isolated from the mycelium of the Lingzhi and/or Reishi medicinal mushroom *Ganoderma lucidum* exhibits anticonvulsant and neuroprotective effects (Tello et al., 2013). The chemical constituents from *H. erinaceus* can stimulate NGF-mediated neurite outgrowth (Phan et al., 2013; Zhang et al., 2015), while Erinacine A can act as an anti-neuroinflammatory agent that confers neuroprotection in Parkinson's disease rat model (Kuo et al., 2016). The same compound can ameliorate AD-related pathologies in APPswe/PS1dE9 transgenic mice (Tsai-Teng et al., 2016). Moreover, the polysaccharides extracted from *H. erinaceus* exhibit antioxidant and neuroprotective effects on Aβ-induced neurotoxicity in neurons (Cheng et al., 2016), and are considered a potent immunostimulant for murine bone marrow-derived dendritic cell maturation (Qin et al., 2017). *H. erinaceus* has been shown to delay the onset of age-associated neurodegenerative diseases (Phan et al., 2014; Trovato et al., 2016), and increase mossy fiber-CA3 hippocampal neurotransmission and recognition memory in wild-type mice, when administered as a dietary supplement (Brandalise et al., 2017). Finally, *H. erinaceus* extracts can alter the behavioral rhythm in mice (Furuta et al., 2016) and possess neuroprotective properties in glutamate-damaged differentiated PC12 cells and in an AD mouse model (Zhang et al., 2016). Based on the aforementioned studies regarding the neuronal effects of medicinal mushrooms, *H. erinaceus* was selected in the present study in order to examine further the pharmacologically active ingredients and corresponding mode of action with regard to the protection against AD. The memory deficit rats were prepared by intraperitoneal injection (i.p) of 100 mg/kg/d D-galactose daily for a total period of 8 weeks, and the water maze tests were used to evaluate the improvement of learning and memory activities. The data indicated that the learning and memory activities were impaired compared with those of the control group ($p < 0.05$), while treatment with the EH could reverse the memory deficits. This finding indicated the identification of certain active ingredients with regard to the treatment of AD in the fruit bodies of *H. erinaceus*.

Beta-site amyloid-β protein precursor cleaving enzyme 1 (BACE1) is the rate limiting enzyme and the initial protease involved in the biosynthetic pathway of amyloid-β (Aβ). BACE1 is considered a potential disease-modifying target for the development of therapeutic drugs for AD (Devraj et al., 2016; Evin, 2016). The research of our group is focused on the screening evaluation of potent BACE1 inhibitors in an effort to identify suitable AD drug candidates. A molecular docking analysis was carried out in order to evaluate the BACE1 inhibitory effect of the compounds from *H. erinaceus*. The molecular docking study was conducted using the LibDock protocol in order to identify potent candidates and investigate receptor–ligand interactions in the Discovery Studio 2.5 (DS2.5) software. The data indicated that the docking score of compounds derived from *H. erinaceus* and the co-crystal ligands were as follows: 211, 180, 176, 169, 164, and 154 for ZPX394, 3-Hydroxyhericenone F (15), Hericenone G (7), Hericenone F (6), Hericerin (12), and Hericene B, respectively. This suggested four compounds with potential activity against BACE1 inhibition. It is tempting to speculate that some of these compounds may the active ingredients of *H. erinaceus* that have demonstrated potent activity for the treatment of AD, although further assays are required to be conducted for such a hypothesis notably for the remaining compounds from *H. erinaceus,* such as compound 7.

Mitochondria are considered important contributors to the development of several aging-associated diseases via the production of reactive oxygen species. The mitochondrial free-radical theory suggests that the progressive alteration of mitochondria that occurs during the aging process results in the increased production of ROS that in turn causes further mitochondrial dysfunction and damage to the cell (López-Otín et al., 2013). The most common paradigm of the failure in mitochondrial electron transport chain (ETC) enzymes reported in AD is the cytochrome c oxidase (COX, complex IV). The activity of cytochrome c oxidase is deficient in different brain regions, in particular in the cerebral cortex and hippocampus (Parker et al., 1994a,b; Fukui et al., 2007). Certain studies further demonstrated that COX inhibition leads to tau hyperphosphorylation and Aβ deposition, whereas the activity of COX is inhibited by the increased levels of Aβ that eventually results in cell death (Cassarino and Bennett, 1999; Eckert et al., 2010; Silva et al., 2011). In the present study, the levels of intracellular ROS were apparently induced, following treatment of 0.03 mM NaN_3 for 24 h, which resulted in a change in the mitochondrial membrane potential and calcium ions ($[Ca^{2+}]_i$) influx. As a consequence, mitochondrial dysfunction occurred. When the cells were pretreated with 3HF at the concentrations of 0.05, 1.00, and 1.50 μg/ml for 2 h and subsequently exposed to 0.03 mM of NaN_3, the aforementioned changes returned to normal levels, which indicated that 3HF can reverse and/or alleviate the NaN_3-induced oxidative damage in PC12 cells.

Calcium ions (Ca^{2+}) play an important role in normal neurotransmission, long- and short-term plasticity, and regulation of gene transcription. The disturbance in Ca^{2+} homeostasis potentiates excitotoxiciticy. Previous studies have demonstrated that the intracellular Ca^{2+} overload promotes cytochrome C release from the mitochondria, via the activation of the NF-κB pathway and the induction of caspase-9 and caspase-3

expression. These processes ultimately lead to cellular apoptosis (Chen et al., 2013; Li J. et al., 2014; Shi et al., 2014). In the present study, 3HF was shown to reduce Ca^{2+} overload and reversed the levels of p21, NF-κB p65, Caspase-9, Caspase-3, and PARP1 proteins. The latter findings demonstrated that 3HF inhibited cell apoptosis via the calcium channel.

Oxidative stress plays a major role in the development of neurodegenerative disorders (Islam, 2017). The age and various genetic and environmental risk factors cause an imbalance in the oxidative-redox system, while the levels of ROS are increased, which in turn stimulates pro-inflammatory gene transcription and release of cytokines and chemokines, such as IL-1, IL-6, and TNF-α. This eventually leads to the neuroinflammatory processes (Prasad, 2016). Chronic neuroinflammation is responsible for the loss of neurons. The levels of the cytokines IFN-γ, IL-1β, IL-17α, and TNF-α in the serum (**Figure 1B**) of D-galactose-induced rats were increased compared with those of control rats, while administration with EH decreased the cytokine levels. The extent of reduction correlated to the doses, whereas EH further decreased the production of ROS in NaN_3-induced oxidative damaged cells, which indicated that it can improve the inflammation environment via the regulation of ROS levels.

Hyperphosphorylation of tau is involved in the formation of neurofibrillar tangles and is a central biochemical event in the pathogenesis of AD (Lee et al., 2001). Tau is a phosphoprotein with 80 potential serine/threonine and five tyrosine phosphorylation sites (Hanger et al., 2009; Wang et al., 2013). A number of sites, including $Ser^{396/404}$, have been associated with neurons in the "pre-tangle" stadium in the brains derived from AD subjects (Bonda et al., 2011; Vingtdeux et al., 2011; Hu et al., 2013). Over-expression of p25 that results from calpain cleavage of p35, has been suggested to be involved in the hyperphosphorylation of tau protein (Camins et al., 2006) and in the formation of Aβ in AD (Liu et al., 2003), possibly coordinating the action of BACE1. BACE1 levels are raised in AD and potentially accelerate the initiating event for the production of Aβ (Liang et al., 2010). Previous studies have demonstrated that hericenones isolated from the fruit body of *H. erinaceus* promoted NGF biosynthesis in rodent cultured astrocytes (Kawagishia et al., 1991; Ma et al., 2010). The results reported in the present study are in agreement with these findings and the compounds in *H. erinaceus* were identified as promising naturally occurring chemical constituents worthy of further development for the pharmaceutical therapy of AD. In the current study, the expression of BACE1 and p-Tau were decreased following treatment with different concentrations of 3HF in NaN_3-induced oxidative damaged cells (**Figure 4**), whereas EH further decreased the expression of tau and $A\beta_{42}$ in the brain of the D-galactose-induced rat model (**Figure 2**).

The present study provides considerable information regarding the use of *H. erinaceus* for the treatment of various neurological diseases, and highlights 3HF as a promising naturally occurring chemical constituent for the therapeutic intervention of AD via the inhibition of the enzyme β-secretase. Although further studies are required to fully validate the efficacy of this compound in the treatment of AD, the data reported indicate that 3HF can ameliorate neuronal damage by reversing the decreased levels of $[Ca^{2+}]_i$ and ROS and improve mitochondrial function, via the increase in mitochondrial membrane potential and ATP levels of the mitochondrial respiratory chain complexes. The aforementioned processes result in decreased expression levels of the AD intracellular markers BACE1, p-Tau, and $A\beta_{42}$.

AUTHOR CONTRIBUTIONS

CD, YT, YJ, ZC, SO, and XY: conceived and designed the experiments. CD, ZC, YJ, and SO: performed the experiments. CD, ZC, YT, and YJ: analyzed the data. CD and YT: wrote the paper and edited the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

The present work was supported by the financial support from the China National Ministry of Science and Technology Plan Projects (2013BAD16B00), the Guangdong Science and Technology Plan Projects (2016A050502032), the Guangzhou Science and Technology Plan Projects (201504281708257), the Guangzhou Science and Technology Plan Projects (201604020009) and the Nanyue Microbial Talents Cultivation Fund of Guangdong Institute of Microbiology.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 12 October 2017
doi: 10.3389/fphar.2017.00709

The Histamine H3 Receptor Antagonist E159 Reverses Memory Deficits Induced by Dizocilpine in Passive Avoidance and Novel Object Recognition Paradigm in Rats

Alaa Alachkar[1], Dorota Łażewska[2], Katarzyna Kieć-Kononowicz[2†] and Bassem Sadek[1†]*

[1] Department of Pharmacology and Therapeutics, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, United Arab Emirates, [2] Department of Technology and Biotechnology of Drugs, Faculty of Pharmacy, Jagiellonian University Medical College, Kraków, Poland

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Edited by:
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Polish Academy of Sciences, Poland
Alfredo Meneses,
Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico
Nikolaos Pitsikas,
University of Thessaly, Greece

***Correspondence:**
Bassem Sadek
bassem.sadek@uaeu.ac.ae

[†] These authors have contributed equally to this work.

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 22 June 2017
Accepted: 21 September 2017
Published: 12 October 2017

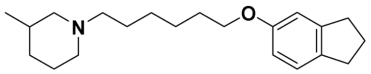
Citation:
Alachkar A, Łażewska D, Kieć-Kononowicz K and Sadek B (2017) The Histamine H3 Receptor Antagonist E159 Reverses Memory Deficits Induced by Dizocilpine in Passive Avoidance and Novel Object Recognition Paradigm in Rats. Front. Pharmacol. 8:709. doi: 10.3389/fphar.2017.00709

The involvement of histamine H3 receptors (H3Rs) in memory is well known, and the potential of H3R antagonists in therapeutic management of neuropsychiatric diseases, e.g., Alzheimer disease (AD) is well established. Therefore, the effects of histamine H3 receptor (H3R) antagonist E159 (2.5–10 mg/kg, i.p.) in adult male rats on dizocilpine (DIZ)-induced memory deficits were studied in passive avoidance paradigm (PAP) and in novel object recognition (NOR) using pitolisant (PIT) and donepezil (DOZ) as standard drugs. Upon acute systemic pretreatment of E159 at three different doses, namely 2.5, 5, and 10 mg/kg, i.p., 2.5 and 5 but not 10 mg/kg of E159 counteracted the DIZ (0.1 mg)-induced memory deficits, and this E159 (2.5 mg)-elicited memory-improving effects in DIZ-induced amnesic model were moderately abrogated after acute systemic administration of scopolamine (SCO), H2R antagonist zolantidine (ZOL), but not with H1R antagonist pyrilamine to the animals. Moreover, the observed memory-enhancing effects of E159 (2.5 mg/kg, i.p.) were strongly abrogated when animals were administered with a combination of SCO and ZOL. Furthermore, the E159 (2.5 mg)-provided significant memory-improving effect of in DIZ-induced short-term memory (STM) impairment in NOR was comparable to the DOZ-provided memory-enhancing effect, and was abolished when animals were injected with the CNS-penetrant histamine H3R agonist *R*-(α)-methylhistamine (RAMH). However, E159 at a dose of 2.5 mg/kg failed to exhibit procognitive effect on DIZ-induced long-term memory (LTM) in NOR. Furthermore, the results observed revealed that E159 (2.5 mg/kg) did not alter anxiety levels and locomotor activity of animals naive to elevated-plus maze (EPM), demonstrating that improved performances with E159 (2.5 mg/kg) in PAP or NOR are unrelated to changes in emotional responding or in spontaneous locomotor activity. These results provide evidence for the potential of drugs targeting H3Rs for the treatment of neuropsychiatric disorders, e.g., AD.

Keywords: histamine H3 receptor, antagonist, learning, memory impairment, passive avoidance paradigm, novel object recognition, elevated plus maze

INTRODUCTION

The main representative character of AD as a neurogenerative disease and related dementias, e.g., cognitive deficit associated with schizophrenia (CDS), is the progressive decline in cognitive performance (Medhurst et al., 2007, 2009; Silva et al., 2014), and enhancing cognitive functions in these conditions embodies a multifaceted task, given the fact that various brain neurotransmission systems and several brain regions are involved in the progress of these conditions (Khan et al., 2015; Shimizu et al., 2015; Sadek et al., 2016a,c). Current pharmacological interventions for AD, such as cholinesterase inhibitors, provide only shortly timed marginal clinical benefit (Silva et al., 2014). Hence, the difficulties to develop satisfactory therapies of AD and CDS are still restricted due to the complicated pathophysiology of these diseases including several pathways, e.g., defective β-amyloid protein metabolism and abnormalities in central neurotransmissions for acetylcholine, glutamate, noradrenaline, serotonin, and dopamine, and the association of these diseases with inflammatory and/or oxidative and hormonal pathways (Doraiswamy, 2002; Cavalli et al., 2008; Zhou et al., 2016). Importantly, the brain histaminergic system's role in AD has been proposed, and a variety of pharmaceutical agents targeting central histaminergic systems have been developed (Bishara, 2010; Baronio et al., 2014; Sadek and Stark, 2015; Sadek et al., 2016c). Accordingly, H3Rs functioning as auto-receptors modulate synthesis and release of central histamine (Sadek and Stark, 2015; Sadek et al., 2016c). Moreover, dysregulations in a wide range of different central neurotransmitter systems, e.g., dopamine, serotonin, GABA, and glutamate were generally hypothesized for the H3Rs located on neurons other than histmainergic neural cells and functioning as hetero-receptors (Witkin and Nelson, 2004; Sadek and Stark, 2015; Sadek et al., 2016c). Notably, H3R antagonists/inverse agonists have been found to exhibit a unique feature by their potential cognition-enhancing property as indicated by numerous lines of evidence from preclinical studies. Accordingly, several H3R antagonists/inverse agonists have been previously found to counteract DIZ-induced memory deficits in rodents (Witkin and Nelson, 2004; Passani and Blandina, 2011; Sadek and Stark, 2015; Sadek et al., 2016c) Furthermore, previous preclinical as well as clinical experiments revealed that antagonists at *N*-methyl-D-aspartate receptors (NMDARs), e.g., ketamine, promote cognitive deficits in healthy humans and exaggerate symptomatic parameters in patients with schizophrenia (Luby et al., 1959; Ghoneim et al., 1985; Javitt and Zukin, 1991; Krystal et al., 1994; Malhotra et al., 1997; Brown et al., 2013). NMDAR antagonists were, also, found to induce behavioral deficits in rodents through impairment of their neurocognitive functions (Large, 2007). In addition, there are several evidences that central histamine significantly alter cognitive deficits and that antagonists/inverse agonists selectively targeting central histamine H3Rs may possibly lead to therapeutic entities with potential clinical use in cognitive symptoms, e.g., AD (Witkin and Nelson, 2004; Esbenshade et al., 2008; von Coburg et al., 2009; Sadek and Stark, 2015; Sadek et al., 2016c). Notably, numerous developed H3R antagonists were revealed in their effects to decrease ketamine- and DIZ-induced cognitive deficits in several animal models of schizophrenia (Browman et al., 2004), signifying that these drugs may also be effective against CDS (Witkin and Nelson, 2004; Bardgett et al., 2010; Charlier et al., 2013; Sadek and Stark, 2015; Sadek et al., 2016c). Moreover, previous preclinical experiments showed that several H3R antagonists, e.g., ABT-239 and A-431404, significantly reduced ketamine- and DIZ-induced cognitive deficits in rats when compared to standard antipsychotics, e.g., olanzapine and risperidone (Brown et al., 2013). Based on the high attention level generated by these preclinical outcomes, the central H3Rs represent an attractive target for developing novel H3R antagonists/inverse agonists with the potential role in neuropsychiatric multi-neurotransmitter disorders, e.g., AD and CDS (Yokoyama et al., 1993; Yokoyama, 2001; Harada et al., 2004; Witkin and Nelson, 2004; Uma Devi et al., 2010; Bhowmik et al., 2012; Khan et al., 2015; Sadek and Stark, 2015; Sadek et al., 2016a,c). Therefore, the effects of the newly developed highly potent and selective non-imidazole H3R antagonist/inverse agonist, E159 [1-(6-(2,3-dihydro-1*H*-inden-5-yloxy)hexyl)-3-methylpiperidine], with high *in vitro* selectivity toward H3Rs (Lazewska et al., 2006) (**Figure 1**) has been investigated on its behavioral effects on DIZ-induced memory deficits in PAP and NOR tasks in adult male rats. Also and since anxiety and motor activity could confound learning and memory's performance of animals (Sadek et al., 2016e), the effects of E159 on locomotor activity and anxiety-like behaviors of the same animals in EPM were tested. Moreover, the abrogative effects of PYR, ZOL, and SCO on the E159-provided memory-enhancing effects in PAP and NOR tests were assessed.



E159

[1-(6-(2,3-dihydro-1*H*-inden-5-yloxy)hexyl)-3-methylpiperidine]

$pK_i(hH3R)^a = 6.12$; $pK_i(hH4R)^b > 4$; $pK_i(hH1R)^c = 4.77$

FIGURE 1 | Chemical structure and *in vitro* affinities of the non-imidazole H3R antagonist/inverse agonist E159. [a] [^{125}I]Iodoproxyfan binding assay at human H3Rstably expressed in CHO-K1 cells, $n = 3$ (Ligneau et al., 1994; Ligneau et al., 2000; Lazewska et al., 2006). [b] [^{3}H]Histamine binding assay performed with cell membrane preparation of Sf9 cells transiently expressing the human histamine H4Rand co-expressed with $G_{\alpha i2}$ and Gβ1γ2 subunits, $n = 3$ (Meier et al., 2001; Amon et al., 2007; Isensee et al., 2009; Tomasch et al., 2013). [c] [^{3}H]Pyrilamine binding assay performed with cell membrane preparation of CHO-hH1Rcells stably expressing the human H1R, $n = 3$ (Schibli and Schubiger, 2002; van Staveren and Metzler-Nolte, 2004; Schlotter et al., 2005).

Abbreviations: AD, Alzheimer disease; CDS, cognitive deficits associated with schizophrenia; DIZ, dizocilpine; DOZ, donepezil; DZP, diazepam; EPM, elevated plus maze; H3Rs, histamine H3 receptors; LTM, long-term memory; NOR, novel object recognition; PAP, passive avoidance paradigm; PIT, pitolisant; PYR, pyrilamine; RAMH, *R*-(α)-methyl-histamine; SCO, scopolamine; STL, step-through latency; STM, short-term memory; ZOL, zolantidine.

MATERIALS AND METHODS

Animals

Inbred male Wistar rats aged 6–8 weeks (body weight: 180–220 g, Central Animal Facility of the UAE University) were maintained in an air-conditioned animal facility room with controlled temperature (24°C ± 2°C) and humidity (55% ± 15%) under a 12-h light/dark cycle. The animals were given free access to food and water. All experimental procedures were conducted between 9:00 and 14:00 h. The procedures used to assess effects of E159 were approved by the Institutional Animal Ethics Committee of CMHS/UAEU(A30-13). All efforts were considered to reduce number of animals used and their suffering. Also, all behavioral studies were conducted by the same experimenter.

Drugs

RAMH dihydrochloride, H1R antagonist PYR, H2R antagonist ZOL dimaleate, DOZ hydrochloride, DIZ hydrogen maleate, and SCO hydrobromide were obtained from Sigma–Aldrich (St. Louis, MO, United States). Chemical synthesis, analysis, and approval of the structure for E159 [1-(6-(2,3-dihydro-1*H*-inden-5-yloxy)hexyl)-3-methylpiperidine] and PIT were conducted in the Department of Technology and Biotechnology of Drugs (Kraków, Poland) as described previously (Lazewska et al., 2006). DZP manufactured by Gulf Pharmaceutical Industries (Ras Al Khaimah, United Arab Emirates) was obtained from Dr. Ameen Al Amaydah (Department of Emergency Medicine, Emirates International Hospital, Al Ain, United Arab Emirates). All doses were expressed in terms of the free base of all drugs. The drugs used in the current study were dissolved in saline and injected i.p. at a volume of 1 ml/kg. All experimental procedures were carried out in a blinded fashion in which the experimenter was uninformed about the specific treatment groups to which an animal group belonged.

Behavioral Tests

Step-Through PAP Test

The step-through PAP test was done as previously described and in an automatically operated commercial Passive Avoidance Apparatus (step-through cage, 7550, Ugo Basile, Comerio, Italy) (Bernaerts et al., 2004; da Silva et al., 2009; Goshadrou et al., 2013; Khan et al., 2015; Sadek et al., 2015, 2016a,e). The experimental procedure consisted of two trials (training and testing) separated by a 24 h interval. Each rat in the first trial was placed in the white compartment, facing the auto guillotine door and, after a 30-s habituation period, the door was raised automatically, and cut-off time of 60 s was given for the animal to cross to the dark compartment. As soon as the rat placed all four paws in the dark module, the guillotine door was lowered and a scrambled foot-shock of 0.4 mA (20 Hz, 8.3 ms) was delivered to the grid floor for 3 s. Rats that failed to move within this period were excepted from the test on the following day. Directly after receiving the shock, the rat was removed from the dark chamber, returned to its home-cage, and the chambers were thoroughly cleaned. For the second and third training day, the same procedure was followed with the only change that a 300 s cut-off latency was allowed for the test animal to enter the dark compartment, however, without delivery of scrambled foot-shock. Animals that failed to cross into the dark compartment during the training, despite the practices conducted in training sessions, were excluded from the current study. For each experiment, 9–11 rats having the same average of age and weight were trained on the step-through latency (STL) test. Approximately 2–4 rats failed to show improved performance in a cut-off time of 60 s, a time period provided for the animals to cross to the dark compartment. In the current experiments, a sample group of seven rats was used for each STL experiment conducted for the PAP. In the test session, animals were turned amnesic with SCO (2 mg/kg) or DIZ (0.1 mg/kg) 30–45 min prior to the test session, and the rats were given a maximum of 300 s to move into the dark box. In this test session, the STL time taken by the animal to enter the dark box or STL in 5 min was recorded and documented. In order to identify a procognitive effect, 11 groups were acutely pretreated with Saline + Saline, DIZ (0.1 mg) + Saline, DIZ (0.1 mg) + E159 (2.5 mg), DIZ (0.1 mg) + E159 (5 mg), DIZ (0.1 mg) + E159 (10 mg), DIZ + DOZ (1 mg), or DIZ + PIT (10 mg) 30–45 min prior to the test session, respectively, and their effects on DIZ-induced memory deficits were measured by determining the STLs to enter the dark box. The E159-provided procognitive effect was confirmed by conducting additional experiments in which the respective promising dose of E159 (2.5 mg/kg) and PYR (10 mg/kg), ZOL (10 mg/kg), SCO (1 mg/kg), or a combination of SCO and ZOL were co-injected. The doses of SCO, PYR, and ZOL were selected according to previous studies (Orsetti et al., 2001, 2002; Khan et al., 2015; Sadek et al., 2015, 2016a,b,d) (**Figures 2–4**).

NOR Test

Novel Object Recognition was assessed with a slight modification as previously described (Ennaceur and Delacour, 1988; Izquierdo et al., 1999; de Lima et al., 2005; Karasawa et al., 2008). The experiments were conducted in a black open field box measuring 50 cm × 35 cm × 50 cm. The experimental procedure included two sessions of habituation separated with a 1-h interval, whereby the animals were permitted for exploratory time of 3-min. On the test day, animals were placed in the test box, and after a 3-min of exploration, two objects (9 cm × 5 cm × 9 cm wood blocks which were in duplicate of the same size but different shape and color) were presented in two corners (approximately 30 cm apart from each other). The experimental session (24 h later) consisted of two trials, namely T1 and T2, each of a duration of 3 min. In T1, rats were exposed to two identical objects, and rats which explored the objects for less than 10 s during T1 were excluded from the experiments. In T2, performed 120 min for STM or 24 h for LTM later, animals were exposed to two objects, one of which was replaced with a new object and the other object was a duplicate of the familiar one to exclude olfactory traits. Also, the familiar or new object as well as the relative position of the two objects were counterbalanced and randomly permuted during trial T2. The measurement was obtained with the time spent by the animal exploring both objects during T1 and T2, and exploration of an object was defined as touching one of both objects with the nose. Other

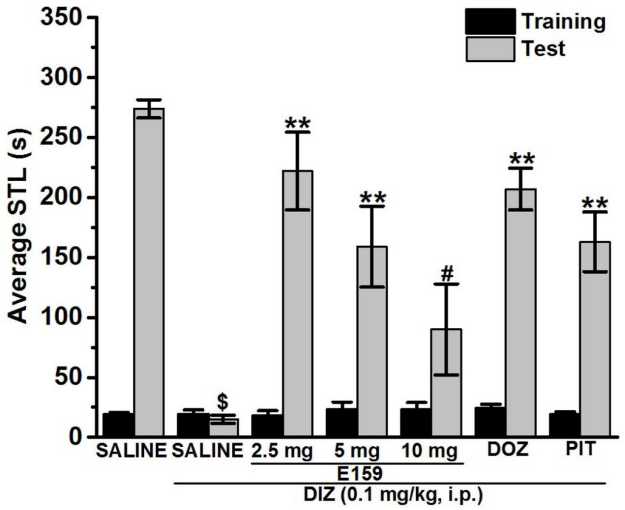


FIGURE 2 | Effects of E159 on DIZ-induced memory deficits in an inhibitory avoidance conditioned response in rats. Gray columns represent the mean STLs measured during the retention test (test latencies) and black columns the mean STLs measured during the training trial before the delivery of the foot-shock (pre-shock latencies). Rats were injected with E159 (2.5, 5, or 10 mg/kg, i.p.), DOZ (1 mg/kg, i.p.), or PIT (10 mg/kg, i.p.) 30–45 min before the test session. $^{**}P < 0.001$ for mean STLs vs. the value of the (saline)-treated group. $^{\#}P < 0.005$ for mean STLs vs. the value of the [E159 (2.5 mg)]-treated group. $^{\$}P < 0.001$ for mean STLs vs. the value of the (saline)-treated group. Data are expressed as mean ± SEM (n = 7).

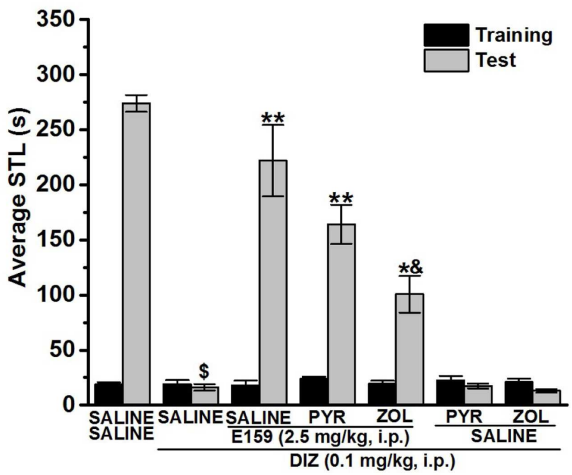


FIGURE 3 | Effect of vehicle, E159, PYR, and ZOL on DIZ-induced deficit in an inhibitory avoidance conditioned response in rats. Gray columns represent the mean STLs measured during the retention test (test latencies) and black columns the mean STLs measured during the training trial before the delivery of the foot-shock (pre-shock latencies). Rats were injected with E159 (2.5 mg/kg, i.p.), PYR (10 mg/kg, i.p.) or ZOL (10 mg/kg, i.p.) 30–45 min before the test session. $^{\$}P < 0.001$ for mean STLs vs. the value of the (Saline)-treated group. $^{**}P < 0.001$ for mean STLs vs. the value of the (DIZ)-treated group. $^{*}P < 0.001$ for mean STLs vs. the value of the (DIZ + E159)-treated group. $^{\&}P < 0.05$ vs. the value of the (DIZ)-treated group. Data are expressed as mean ± SEM (n = 7).

behavioral observations, e.g., turning around or sitting on the object was not considered an experimental behavior. The open field arena as well as the objects were carefully cleaned using 70% (volume/volume; v/v) alcohol. DIZ and all test compounds were dissolved in saline and administered i.p. 30 min following T1 at a volume of 1 ml/kg, and the doses were expressed in terms of the free base. The control groups received an equivalent volume of saline. The doses chosen for the NOR test were derived from the results in PAP and/or previously reported procognitive studies (Bernaerts et al., 2004; da Silva et al., 2009; Goshadrou et al., 2013; Khan et al., 2015; Sadek et al., 2015; Sultan et al., 2017). In order to detect a procognitive effect for STM, eight groups of 6–8 rats each were used. They were injected with Saline + Saline, DIZ + Saline, DIZ + E159 (2.5 mg/kg), DIZ + E159 (2.5 mg) + RAMH (10 mg), DIZ + DOZ (1 mg), DIZ + RAMH (10 mg), Saline + E159 (2.5 mg/kg), or Saline + RAMH (10 mg/kg) 30–45 min after T1, and their effects on DIZ-induced cognitive deficits (STM) were measured by determining the time spent by the rat in exploring objects during trials T1 and T2 (**Figure 4**). Moreover, the variable N-F/N + F which provides the discrimination index (D) was computed. Also and in order to exclude any confounding factors, E159 (2.5 mg/kg) and RAMH (10 mg/kg) were tested on their effect on two separate saline-treated control groups. The above mentioned experimental protocol was applied to detect the procognitive effect for LTM in another six groups of 6–8 rats each, however, with the only change that E159 (2.5 mg/kg, i.p.) was administered 30–45 min prior to T2. They were injected with Saline + Saline, DIZ + Saline, DIZ + E159 (2.5 mg/kg), DIZ + DOZ (1 mg), Saline + E159 (2.5 mg/kg), or Saline + DOZ(1 mg/kg) (**Figure 5**). In all experiments, doses of DIZ, RAMH, and DOZ were selected according to previous studies (Bernaerts et al., 2004; de Lima et al., 2005; da Silva et al., 2009; Goshadrou et al., 2013; Khan et al., 2015; Sadek et al., 2015, 2016a; Sultan et al., 2017) (**Figures 5**, **6**).

EPM Test

Anxiety-like behaviors were with slight modification assessed in an EPM as previously described (Jiang et al., 2016). The EPM apparatus consisted of several parts including one central part (8 cm × 8 cm), two opposing open and closed arms (30 cm × 8 cm), and non-transparent walls (30 cm in height). The experiment room was light and temperature-controlled, and both the plat form and the wall were thoroughly cleaned between every session using 10% ethanol spray. Animals were placed individually in the center arena of the maze (50 cm above the floor) facing the open arm. The measurement was carried out by observing the amount of time spent with head and forepaws on the open arms and closed arms of the maze and the number of entries into each arm was manually scored for a duration of 5 min. In this experiment, the total number of entries into the closed arms is typically considered as an index for locomotor activity of the respective tested animal. In order to detect anxiety-like and locomotor behavior, three groups of 8–10 animals each were injected i.p. with saline (Saline group) and two test groups

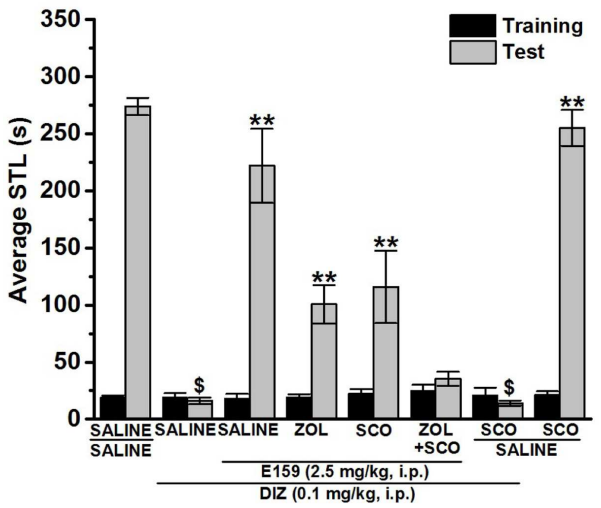


FIGURE 4 | Effect of vehicle, E159, ZOL, and SCO on DIZ-induced deficit in an inhibitory avoidance conditioned response in rats. Gray columns represent the mean STLs measured during the retention test (test latencies) and black columns the mean STLs measured during the training trial before the delivery of the foot-shock (pre-shock latencies). Rats were injected with E159 (2.5 mg/kg, i.p.), ZOL (10 mg/kg, i.p.), SCO (1 mg/kg, i.p.) or a combination of ZOL (10 mg/kg) + SCO (1 mg/kg) 30–45 min before the test session. $^{\$}P < 0.001$ for mean STLs vs. the value of the (Saline)-treated group. $^{**}P < 0.001$ for mean STLs vs. the value of the (DIZ)-treated group. $^{\#}P < 0.05$ for mean STLs vs. the value of the (DIZ + E159 + ZOL)- and (DIZ + E159 + SCO)-treated group vs. the value of the (DIZ)-treated group. Data are expressed as mean ± SEM (n = 7).

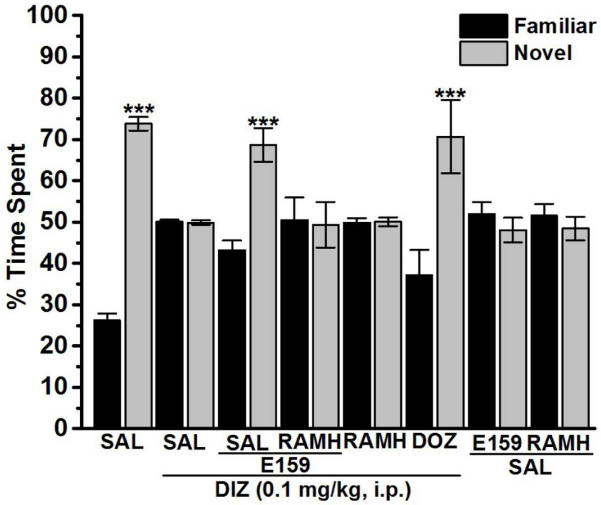


FIGURE 5 | Effects of E159 on DIZ-induced STM cognitive deficits in the object recognition test in rats. Thirty minutes following training session T1, E159 (2.5 mg/kg), RAMH (10 mg/kg), DOZ (1 mg/kg), or DIZ (0.1 mg/kg) was administrated intraperitoneally. The test session T2 was performed 120 min (STM) after the training session T1. Results are calculated as individual percentage of time spent exploring familiar (black columns) and novel (gray columns) objects. Data represent mean ± SEM of 6–8 animals per experimental group. $^{***}P < 0.001$ vs. respective familiar object.

that received either E159 (2.5 mg/kg) or DZP (10 mg/kg) (**Figures 7A–D**).

Statistical Analysis

IBM® SPSS Statistics® version 20 software (IBM Middle East, Dubai, United Arab Emirates) was used for all statistical comparisons in all behavioral experiments. Data were expressed as means ± SEM. The effects of E159 on DIZ-induced memory deficits were analyzed using a two-way analysis of variance (ANOVA) with Treatment (vehicle or E159) and Dose (E159) as the between-subjects factor. The effect of E159 in combination with PYR, ZOL, or SCO on STL time was analyzed using one-way ANOVA with Treatment as the between-subject factor. The effects of E159 with the most promising dose in PAP (2.5 mg/kg) on DIZ-induced amnesia in NOR test were analyzed using a mixed repeated-measure two-way analysis of variance (ANOVA) with Treatment (vehicle or E159) and Dose (E159; 2.5 mg/kg, i.p.) as the between-subjects factor. The effect of E159 (2.5 mg/kg) in EPM test were assessed by measuring the time spent on the open arms and closed arms of the maze as well as the number of entries into each arm. The results observed in EPM test were analyzed using one-way ANOVA with Treatment as the between-subject factor. In case of a significant main effect, *post hoc* comparisons were performed with Bonferroni's test. The criterion for statistical significance was set at $p \leq 0.05$.

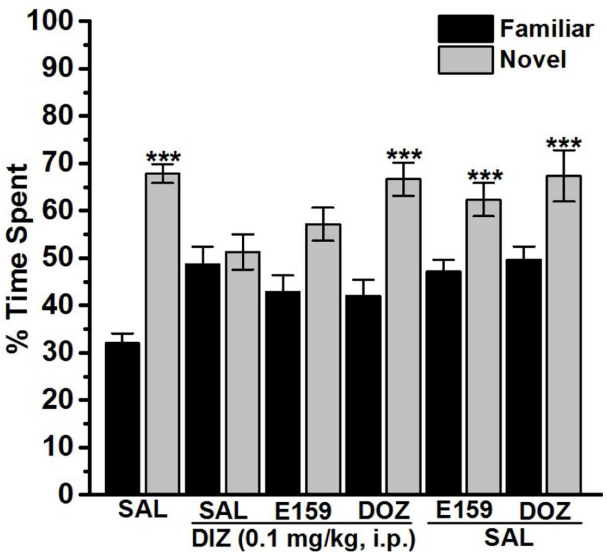


FIGURE 6 | Effects of E159 on DIZ-induced LTM cognitive deficits in the object recognition test in rats. Thirty minutes following training session T1, E159 (2.5 mg/kg), DOZ (1 mg/kg), or DIZ (0.1 mg/kg) was administrated intraperitoneally. The test session T2 was performed 24 h (LTM) after the training session T1 in which E159 (2.5 mg/kg) was administered 30–45 min before T2. Results are calculated as individual percentage of time spent exploring familiar (black columns) and novel (gray columns) objects. Data represent mean ± SEM of 6–8 animals per experimental group. $^{***}P < 0.001$ vs. respective familiar object.

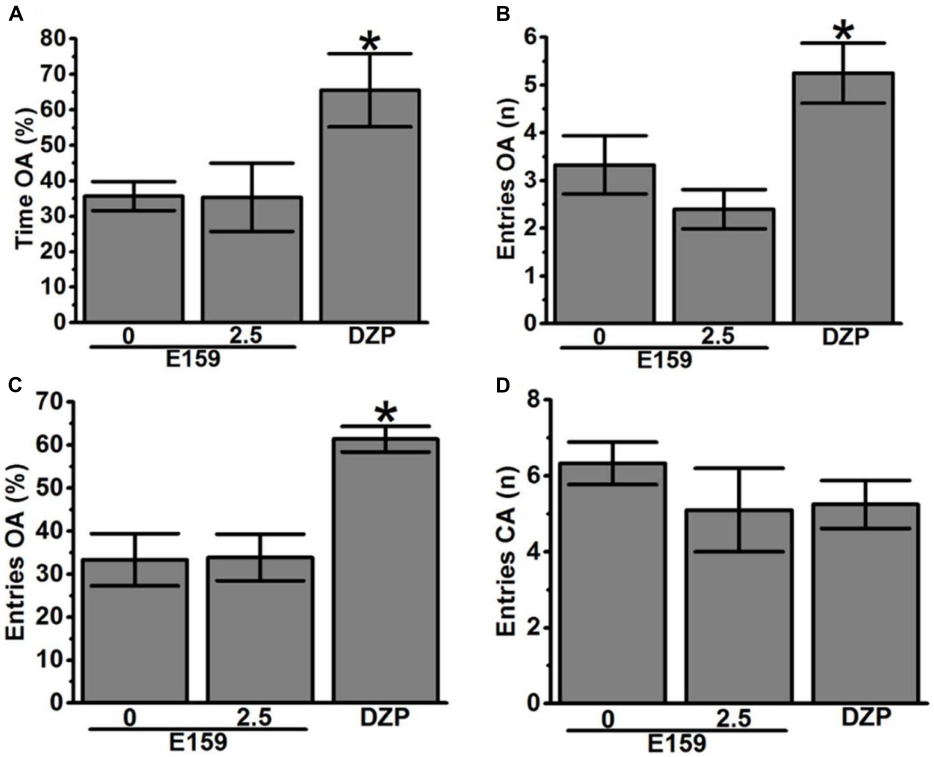


FIGURE 7 | Effects of acute E159 pretreatment on exploratory behavior in the EPM test. E159 (0 or 2.5 mg/kg) did not change the percentage of time spent on the open arms of the EPM **(A)**, the number of entries into the open arms **(B)**, and the percentage of entries into the open arms **(C)**. Pretreatment with the H3R antagonist/inverse agonist E159 did not affect the number of closed arm entries **(D)**. However, DZP significantly increased the percentage of time spent on the open arms of the EPM **(A)**, increased the number of entries into the open arms **(B)** and the percentage of entries into the open arms **(C)**, without significant alteration in the number of closed arm entries **(D)**. Data are expressed as mean ± SEM (n = 8–10). *$P < 0.05$ for the value of DZP-treated group vs. the value of the E159 (Saline)- or (2.5 mg)-treated group.

RESULTS

Effects of E159, DOZ, and PIT on DIZ-Induced Memory Impairment in Step-Through PAP

Figure 2 shows the effect of E159 (2.5, 5, and 10 mg/kg), DOZ (1 mg/kg), and PIT (10 mg/kg) on DIZ-induced memory impairments in step-through PAP in rats. When injected before the retention test, one-way analysis of variance showed that acute systemic prtreatment with E159 (2.5, 5, and 10 mg/kg), DOZ (1 mg/kg), and PIT (10 mg/kg) exihbited a significant effect on STLs [$F_{(13,84)} = 9.691$; $P < 0.001$]. Also and as compared to the (saline)-treated group, subsequent *post hoc* analyses showed that DIZ (0.1 mg/kg) decreased STL time with [$F_{(1,12)} = 820.401$; $P < 0.001$]. In addition, E159 (2.5 and 5 mg/kg), DOZ (1 mg/kg), and PIT (10 mg/kg) exerted significant memory-enhancing effect on STLs when compared to (DIZ)-treated group with [$F_{(1,12)} = 34.631$; $P < 0.001$], [$F_{(1,12)} = 15.392$; $P < 0.001$], [$F_{(1,12)} = 95.365$; $P < 0.001$], and [$F_{(1,12)} = 30.074$; $P < 0.001$], respectively. The procognitive effects observed for E159 at a dose of 2.5 mg/kg were not significantly different from (Saline)-treated rats ($p = 0.1703$). However, E159 tested at a dose of 10 mg/kg failed to exhibit significant memory enhancing effect when compared to (DIZ)-treated group ($p = 0.093$).

Effects of PYR and ZOL on the E159-Provided Memory Improvement in DIZ-Induced Deficit in Step-Through PAP

For this experiment, separate groups of rats ($n = 7$ for each group) were pretreated with either the CNS-penetrant H1R antagonist PYR (10 mg/kg) or the CNS-penetrant H2R antagonist ZOL (10 mg/kg) 30–45 min prior to the test session. As shown in **Figure 3**, pairwise comparisons reported that, as expected, acute systemic pretreatment with E159 (2.5 mg/kg, i.p.) prolonged STL time when compared to the (DIZ)-treated group with [$F_{(1,12)} = 34.346$; $P < 0.001$]. This E159-provided prolongation of STL time was partly abrogated following ZOL [$F_{(1,12)} = 9.437$; $p = 0.009$: Saline + DIZ + E159 vs. DIZ + E159 + ZOL], but not following an acute systemic administration with PYR when compared to (Saline + DIZ + E159)-treated group [$F_{(1,12)} = 2.082$; $p = 0.175$]. Notably, neither Saline + Saline + DIZ vs. Saline + DIZ + PYR, nor Saline + Saline + Saline vs. Saline + DIZ + ZOL differences were found to be significant ($p = 0.70$ and $p = 0.47$, respectively) (**Figure 3**).

Effects of ZOL, SCO, and a Combination of ZOL and SCO on the E159-Provided Memory-Improvement in DIZ-Induced Deficit in Step-Through PAP

As depicted in **Figure 4**, one-way analysis of variance showed that acute systemic pretest administration with E159 (2.5 mg/kg), ZOL (10 mg/kg), SCO (1 mg/kg), and combination of ZOL (10 mg/kg) with SCO (1 mg/kg) exerted a significant memory improving effect on STLs [$F_{(7,48)} = 29.436$; $P < 0.001$]. Moreover, subsequent *post hoc* analyses revealed that DIZ (0.1 mg/kg) reduced STL time with [$F_{(1,12)} = 849.171$; $P < 0.001$] when compared to the (Saline)-treated group (**Figure 4**). Furthermore, E159 (2.5 mg/kg) exhibited significant improving effect on STLs with [$F_{(1,12)} = 34.346$; $P < 0.001$] when compared to (DIZ)-treated group, and this E159-induced improvement of STL time was not completely abrogated following acute systemic co-administration with ZOL (10 mg/kg) or SCO (1 mg/kg) with [$F_{(1,12)} = 21.382$; $P < 0.001$: Saline + Saline + DIZ vs. DIZ + E159 + ZOL] and [$F_{(1,12)} = 8.429$; $P < 0.05$: Saline + Saline + DIZ vs. DIZ + E159 + SCO]. In addition, an acute systemic pretreatment with ZOL (10 mg/kg) combined with SCO (1 mg/kg) showed significantly higher abrogative effect on the E159-provided memory improvement when compared with the abrogation observed by ZOL or SCO administered alone with [$F_{(1,12)} = 11.466$; $P < 0.05$: DIZ + E159 + ZOL vs. DIZ + E159 + ZOL + SCO] and [$F_{(1,12)} = 5.288$; $P < 0.05$: DIZ + E159 + SCO vs. DIZ + E159 + ZOL + SCO], respectively. Interestingly, SCO failed to alter STL time in both (Saline)- and Saline + DIZ-treated group ($p = 0.757$ and $p = 0.334$, respectively) (**Figure 4**).

Effects of E159 and DOZ on DIZ-Induced STM Deficits in NOR

The results observed in NOR test for the total time exploring both objects during training and test session of the respective group revealed that there were no significant differences between (Saline)- and DIZ-treated groups (**Table 1**). The latter observation is important to exclude any confounding factors, e.g., that the acute post-training injection with DIZ in the first experiment did not modulate locomotor activity or motivation as sensorimotor parameters. Also, statistical analyses of results observed for exploratory time during T1 revealed that no significant differences were present in exploration between the two identical objects. **Figure 5** shows the effect of E159 (2.5 mg/kg) and DOZ (1 mg/kg) on DIZ-induced STM deficits of memory in NOR. Moreover, one-way analysis of variance showed that acute systemic pretreatment with E159 (2.5 mg/kg) and DOZ (1 mg/kg) exhibited a significant effect on exploratory time spent with both objects in T2 with [$F_{(7,40)} = 5.799$; $P < 0.001$] when injected 30 min after training session T1. As shown by subsequent *post hoc* tests, DIZ (0.1 mg/kg) decreased memory for the novel object in T2 with [$F_{(1,12)} = 205.423$; $P < 0.001$] when compared to the (saline)-treated group. However, E159 (2.5 mg/kg) enhanced impaired STM when compared to (DIZ)-treated group with [$F_{(1,12)} = 24.396$; $P < 0.001$], and was comparable to the DOZ(1 mg/kg)-provided memory-enhancing effect ($p = 0.706$) (**Figure 5**). Moreover, discrimination indices measured for the different groups in STM support the latter observed results (**Table 1**).

Effects of E159 and DOZ on DIZ-Induced LTM Deficits in NOR

One-way analysis of variance revealed that acute systemic pretreatment with E159 (2.5 mg/kg) and DOZ (1 mg/kg) exerted no significant effect on time spent exploring objects in T2 with [$F_{(5,30)} = 1.293$; $p = 0.293$] when injected 30 min after training session T1 and 60 min before T2 and 24 h later (**Figure 6**). As shown by subsequent *post hoc* analyses, DIZ (0.1 mg/kg) impaired memory for the novel object in T2 with [$F_{(1,10)} = 12.788$; $P < 0.05$] when compared to the (Saline)-treated group (**Figure 6**). Moreover, acute systemic pretreatment with DOZ (1 mg/kg) enhanced LTM with [$F_{(1,10)} = 7.485$; $P < 0.05$] as compared to (DIZ)-treated group. However, E159 (2.5 mg/kg) failed to significantly enhance LTM when compared to (DIZ)-treated group with [$F_{(1,10)} = 1.094$; $p = 0.320$]. Also, acute systemic administration of E159 (2.5 mg/kg) or DOZ (1 mg/kg, i.p.) alone failed to modulate LTM in T2 when compared to the DIZ-treated group with [$F_{(1,10)} = 2.285$; $p = 0.057$] and [$F_{(1,10)} = 0.765$; $p = 0.402$], respectively (**Figure 6**). Also, observed discrimination indices for the different treated groups support the latter results in LTM (**Table 1**).

Effect of E159 on Rat Performance in EPM Test

Figure 7 shows the effects of acute administration of E159 (0 or 2.5 mg/kg) on the percentage of time spent in open arms, number of entries into open arms, percentage entries into open arms, and locomotor activity (number of entries into closed arm) of rats observed in the EPM test. *Post hoc* analyses indicated that compared to saline, E159 failed to alter the percentage of time spent exploring the open arms of the maze during a 5-min session with [$F_{(1,14)} = 0.001$, $p = 0.981$] as compared to the (Saline)-treated group (**Figure 7A**). Moreover, statistical analyses of data describing the number and percentage of entries into the open arms of the maze [$F_{(1,12)} = 1.389$, $p = 0.261$; $F_{(1,10)} = 0.003$, $p = 0.954$, respectively) generated essentially the same results. As shown in **Figures 7B,C**, no significant difference from that obtained with the (Saline)-treated group were observed following acute systemic administration with E159 (2.5 mg/kg). However, the percentage time spent in open arms and number and percentage of entries into open arms were significantly modulated after acute systemic administration of DZP (10 mg/kg, i.p.) with [$F_{(1,12)} = 14.482$, $P < 0.05$], [$F_{(1,10)} = 13.257$, $P < 0.05$], and [$F_{(1,11)} = 15.805$, $P < 0.05$], respectively (**Figures 7A–C**). Interestingly, the number of closed arm entries following E159 (2.5 mg/kg) or DZP (10 mg/kg) injections with [$F_{(1,14)} = 0.554$, $p = 0.469$] and [$F_{(1,12)} = 1.311$, $p = 0.275$] were not significantly changed, indicating that locomotor activity *per se* was not modulated subsequent to acute administrations of E159 or DZP when compared to

TABLE 1 | Effects of E159 on DIZ-induced total amount of time spent exploring both objects during object recognition training and test session in rats.

		Time exploring objects (s)				Discrimination index "*D*"[a]	
Group	***n***	**STM T1**	**STM T2**	**LTM T1**	**LTM T2**	**STM**	**LTM**
Saline	6	37.50 ± 3.20	37.83 ± 3.39	36.67 ± 2.95	39.83 ± 3.22	0.51 ± 0.05	0.36 ± 0.04
DIZ (0.1 mg/kg)	8	36.00 ± 2.73	42.25 ± 1.57	23.33 ± 2.01	22.67 ± 2.35	0.03 ± 0.01*	0.03 ± 0.07*
DIZ + E159 (2.5 mg/kg)	8	23.75 ± 2.18	22.67 ± 4.84	24.63 ± 2.74	27.67 ± 3.48	0.14 ± 0.05#	0.05 ± 0.09
DIZ + E159 (2.5 mg/kg) + RAMH (10 mg/kg)	6	37.50 ± 1.52	41.17 ± 2.43	ND	ND	0.06 ± 0.11	ND
DIZ + DOZ (1 mg/kg)	6	24.83 ± 1.72	25.33 ± 1.68	23.00 ± 3.34	21.00 ± 2.49	0.28 ± 0.11*	0.32 ± 0.07*
DIZ + RAMH (10 mg/kg)	6	23.50 ± 1.41	24.33 ± 1.59	ND	ND	0 ± 0.02	ND
Saline + E159 (2.5 mg/kg)	6	35.67 ± 2.93	37.33 ± 2.19	35.50 ± 2.78	36.83 ± 1.46	0.36 ± 0.07	0.24 ± 0.15
Saline + RAMH (10 mg/kg)	6	35.67 ± 2.93	32.33 ± 1.59	ND	ND	0.34 ± 0.09	ND

Data are expressed as mean ± SEM of 6–10 animals per experimental group. There were no significant differences among training and test exploring time in STM and LTM group of each respective treatment.

[a]*Discrimination index (D) calculated as; D = N − F/N + F, where N and F are the time spent with novel object and familiar object, respectively. ND, not determined.* *$P < 0.05$ *for mean D vs. the value of the (DIZ)-treated group vs. value of (Saline)-treated group.* #$P < 0.05$ *for mean D vs. the value of the (Saline)- and (DIZ + E159)-treated group vs. the value of the (DIZ)-treated group. Data are expressed as mean ± SEM (n = 7).*

that obtained with saline pretreatment (**Figure 7D**). Therefore, the detected behavioral alterations were not influenced by any substantial modifications in the traveled distance during the test period.

DISCUSSION

Mounting evidences show that acute systemic administration of NMDAR antagonists such as DIZ (Luby et al., 1959) reduces performance of experimental rodents in a wide-ranging varieties of learning and memory tasks (Javitt and Zukin, 1991) including PAP and NOR (Luby et al., 1959; Ghoneim et al., 1985; Javitt and Zukin, 1991; Krystal et al., 1994; Malhotra et al., 1997; Brown et al., 2013; Khan et al., 2015; Sadek and Stark, 2015; Sadek et al., 2016a,c). Therefore, DIZ-induced dementia has been commonly used to evaluate potential therapeutic agents for treating AD and CDS (Luby et al., 1959; Ghoneim et al., 1985; Javitt and Zukin, 1991; Krystal et al., 1994; Malhotra et al., 1997; Brown et al., 2013). In this study, acute systemic administration of E159 only at lower doses (2.5 and 5 mg/kg) significantly reversed the DIZ-induced memory deficits in PAP test in adult rats (**Figure 2**). It has been proposed that NMDA receptors participate with a significant function in several stages of memory, namely consolidation and retrieval processes (Vorobjev et al., 1993; Brabant et al., 2009, 2013). Therefore, it is possible that in our experiments E159 moderately reduced DIZ-induced memory deficits through direct stimulation of NMDA receptors by the increased release of central histamine as a consequence of blocking histamine H3 auto-receptors by this class of H3R antagonists. These results are in consensus with earlier observations in which histamine was found to improve transmission in cultured hippocampal cells mediated by NMDA receptors, indicating that the interaction between histamine and NMDA receptors possibly will enable the histamine's capability reduce DIZ-induced memory deficits (Vorobjev et al., 1993; Xu et al., 2005; Brabant et al., 2013; Sadek et al., 2016a). Importantly, the memory-enhancing effect observed for E159 was dose-dependent, since the improvement of memory provided by E159 (2.5 mg/kg) in the DIZ-induced amnesia model was significantly higher when compared to the higher doses (5 and 10 mg/kg), demonstrating that an optimum in memory-enhancing effect was observed when the H3R antagonist/inverse agonist E159 was applied at the lowest dose (2.5 mg/kg), and an off-target effect for E159 at higher doses (5 and 10 mg/kg) might have been observed in the current study (**Figure 2**). The latter observations of dose dependency are, also, in agreement with earlier experimental results conducted in different rodents (Benetti and Izquierdo, 2013; Benetti et al., 2013; Sadek et al., 2015). Moreover, the observed cognitive enhancing effects for E159 (2.5 mg/kg) were similar to those obtained for the reference H3R antagonist/inverse agonist PIT and the reference drug DOZ (**Figure 2**). Furthermore, the E159 (2.5 mg/kg)-provided memory-enhancing effects were moderately reversed when rats were administered with the CNS-penetrant H2R antagonist ZOL but not with the CNS-penetrant H1R antagonist PYR. The latter observation confirmed our previous results observed for the non-imidazole H3R antagonist DL77 (2.5–10 mg/kg) in different memory processes, namely acquisition, consolidation, and retrieval (Sadek et al., 2016e). The ameliorative effects found for E159 in DIZ-induced memory deficits further indicate that histaminergic pathways through activation of H2Rs are fundamentally contributing in neuronal pathways important for alteration of retrieval processes. An additional experiment revealed that the E159-provided memory-enhancing effect was, also, moderately abrogated when animals were administered with SCO, however, significantly further abrogated when animals were administered with a combination of SCO and ZOL (**Figure 3**). The latter experimental finding clearly indicates that cholinergic muscarinic neurotransmission as well as histaminergic circuits through activation of H2Rs are strongly involved in the E159-provided memory enhancing effects (**Figure 3**). Unlike the PAP test, NOR test in rodents measures natural behavior of rodents and advantages from their distinctive curiosity for discovering their surroundings, and it does not comprise a punishment or a reward.

Notably, the NOR is a paradigm used in rodent models to capture characteristics of the neurodevelopmental basis of CDS by interpreting the results without confounding factors, e.g., side effects such as antinociceptive effect of several old-generation imidazole-based H3R antagonists/inverse agonists, e.g., thioperamide (Jaaro-Peled, 2009; Tseng et al., 2009; Brown et al., 2013), and it was found to show high sensitivity to both cognition impairing (Ennaceur and Delacour, 1988; Ennaceur and Meliani, 1992a,b) and enhancing agents (Lebrun et al., 2000; Barak and Weiner, 2011). The results observed in the current study showed that acute systemic post-training administration of E159 (2.5 mg/kg) significantly improved the time spent to explore the novel object compared with the familiar object, and delivered a type of STM (**Figure 5**). These results are in line with earlier observations which revealed that numerous imidazole-based H3R antagonists, e.g., thioperamide and clobenpropit (Giovannini et al., 1999), and non-imidazole based H3R antagonists, e.g., PIT (Ligneau et al., 2007); GSK189254 (Giannoni et al., 2010), SAR110894 (Griebel et al., 2012), and ABT-239 (Provensi et al., 2016) ameliorate the amnesic effects of SCO, DIZ or time in rodents in NOR tests. Interestingly, the STM-enhancing effects provided with E159 in DIZ-induced memory deficits were significantly abrogated after animals were co-injected with the CNS-penetrant H3R agonist RAMH (**Figure 5**). The latter observations are in line with an earlier preclinical study in which RAMH abolished the memory-enhancing effects obtained by the imidazole-based H3R antagonist ciproxifan on STM in mice (Pascoli et al., 2009). Contrary, acute systemic post-training administration of E159 (2.5 mg/kg) failed to increase the time spent exploring the novel objects when compared with the familiar objects in LTM (**Figure 6**). These results in NOR obviously indicate that histaminergic H3Rs are profoundly contributing in neuronal circuits involved in the E159-provided STM-memory enhancing effects, but not in LTM-enhancing effects (**Figure 6**). Moreover, the discrepancies observed for E159 in PAP and NOR might be explained with the differences of conducts and measured features of both models in rodents. Accordingly, NOR measures natural behavior of animals and advantages from their distinctive curiosity for discovering their surroundings, and it does not comprise a punishment or a reward. Also, NOR is a commonly used paradigm in rodents capture characteristics of the neurodevelopmental basis of CDS (Jaaro-Peled, 2009; Tseng et al., 2009; Brown et al., 2013). Interestingly, several H3R antagonists were in previous preclinical studies identified as promising targets for CDS and were proposed to be of potential therapeutic value based on the fact that H3R functions as auto- and hetero-receptor, thus, modulating the biosynthesis and release of several neurotransmitters, including histamine, dopamine, and acetylcholine, which are important for cognitive functions (Brioni et al., 2011; Sadek and Stark, 2015; Sadek et al., 2016c). Notably, E159 at the dose of 2.5 mg/kg, the dose that provided the most promising procognitive effect in PAP and NOR, failed to change anxiety levels and locomotor activity of the tested rodents (**Figures 7A–D**). Moreover, E159 used at the same dose did not alter the number of closed arm entries, indicating that E159 did not change locomotor activity of rodents (**Figure 7D**). The latter observations are significant, since improved performance in PAP or NOR can be the consequence of several variables not related to memory-enhancing effects such as modifications in emotional responding or in spontaneous locomotor activity (McGaugh and Roozendaal, 2009; Charlier et al., 2013). These results are, also, in agreement with previous observations in which acute systemic administration of the H3R antagonist DL77 (2.5–10 mg/kg) failed to modify spontaneous locomotor activity of the same animal species in the open field test (Sadek et al., 2016e).

CONCLUSION

The observed results show that the non-imidazole H3R antagonist E159 reduces DIZ-induced cognitive deficits in PAP and NOR task in adult male rats. Also, the results observed in PAP reveals that acute systemic pretreatment with E159 in DIZ-induced amnesia models significantly ameliorates cognitive impairments via mechanisms dependent on cholinergic muscarinic neurotransmission and – at least partially – H2Rs activation. Moreover, the present results strongly support the potential therapeutic value of histamine H3R antagonists in the treatment of neuropsychiatric diseases, e.g., AD and CDS. Nonetheless, additional preclinical experiments are still warranted with a series of further behavioral test models and with different rodent species to increase the validity of the translational value for possible applicability of H3R antagonists/inverse agonists in the modulation of memory impairment in several neuropsychiatric diseases.

AUTHOR CONTRIBUTIONS

BS was responsible for the study concept, design, acquisition, and analysis of animal data. AA conducted behavioral experiments. KK-K and DŁ were responsible for the generation, synthesis, and pharmacological *in vitro* characterization the H3R antagonist DL77. BS drafted the manuscript. KK-K, DŁ, and AA provided critical revision for the manuscript. All authors critically reviewed content and approved final version for publication.

ACKNOWLEDGMENTS

BS was supported by intermural research grants sponsored by the Research Office of United Arab Emirates University. The authors acknowledge the partial support of Polish National Science Center granted on the basis of decision numbers DEC-2011/02/A/NZ4/00031; K/ZDS/007121. Support was kindly provided by the EU COST Action CA151315 (KK-K and DŁ).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 28 June 2017
doi: 10.3389/fphar.2017.00415

Administration of a Histone Deacetylase Inhibitor into the Basolateral Amygdala Enhances Memory Consolidation, Delays Extinction, and Increases Hippocampal BDNF Levels

*Fernanda E. Valiati[1,2], Mailton Vasconcelos[3], Martina Lichtenfels[2], Fernanda S. Petry[1,2], Rosa M. M. de Almeida[3], Gilberto Schwartsmann[2,4], Nadja Schröder[5], Caroline B. de Farias[2,6] and Rafael Roesler[1,2]**

[1] Department of Pharmacology, Institute for Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [2] Cancer and Neurobiology Laboratory, Experimental Research Center, Clinical Hospital, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [3] Institute of Psychology, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [4] Department of Internal Medicine, Faculty of Medicine, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, [5] Neurobiology and Developmental Biology Laboratory, Faculty of Biosciences, Pontifical Catholic University of Rio Grande do Sul, Porto Alegre, Brazil, [6] Children's Cancer Institute, Porto Alegre, Brazil

OPEN ACCESS

Edited by:
Antonella Gasbarri,
University of L'Aquila, Italy

Reviewed by:
Patrizia Campolongo,
Sapienza Università di Roma, Italy
Christa McIntyre,
University of Texas at Dallas,
United States

***Correspondence:**
Rafael Roesler
rafaelroesler@hcpa.edu.br

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 05 May 2017
Accepted: 13 June 2017
Published: 28 June 2017

Citation:
Valiati FE, Vasconcelos M, Lichtenfels M, Petry FS, de Almeida RMM, Schwartsmann G, Schröder N, de Farias CB and Roesler R (2017) Administration of a Histone Deacetylase Inhibitor into the Basolateral Amygdala Enhances Memory Consolidation, Delays Extinction, and Increases Hippocampal BDNF Levels. Front. Pharmacol. 8:415. doi: 10.3389/fphar.2017.00415

Gene expression related to the formation and modification of memories is regulated epigenetically by chromatin remodeling through histone acetylation. Memory formation and extinction can be enhanced by treatment with inhibitors of histone deacetylases (HDACs). The basolateral amygdala (BLA) is a brain area critically involved in regulating memory for inhibitory avoidance (IA). However, previous studies have not examined the effects of HDAC inhibition in the amygdala on memory for IA. Here we show that infusion of an HDAC inhibitor (HDACi), trichostatin A (TSA), into the BLA, enhanced consolidation of IA memory in rats when given at 1.5, 3, or 6 h posttraining, but not when the drug was infused immediately after training. In addition, intra-BLA administration of TSA immediately after retrieval delayed extinction learning. Moreover, we show that intra-BLA TSA in rats given IA training increased the levels of brain-derived neurotrophic factor in the dorsal hippocampus, but not in the BLA itself. These findings reveal novel aspects of the regulation of fear memory by epigenetic mechanisms in the amygdala.

Keywords: histone deacetylase, brain-derived neurotrophic factor, amygdala, hippocampus, memory extinction, memory consolidation

INTRODUCTION

In inhibitory avoidance (IA), a type of fear-motivated conditioning, a new memory is formed after a single training trial, and the behavioral outcome of previously formed memories can be modified upon recall through extinction or reconsolidation. These processes are mediated and regulated by a range of neurotransmitter and neuropeptide receptors, intracellular protein kinase signaling pathways, and transcription factors, resulting in changes in gene transcription. Brain areas critically

involved in mediating or regulating the formation and extinction of IA memory include the dorsal hippocampus and the basolateral amygdala (BLA; McGaugh, 2000; Roesler and McGaugh, 2010; Roesler and Schröder, 2011; Roozendaal and McGaugh, 2011; Alberini and Kandel, 2014; Furini et al., 2014; Roesler et al., 2014a; Izquierdo et al., 2016). The BLA is proposed to interact with the hippocampus and related structures to enhance the consolidation of memory for events that trigger fear or aversiveness (McGaugh, 2002; McIntyre et al., 2003).

Gene expression related to memory consolidation is regulated epigenetically by chromatin remodeling, post-translational DNA modifications, and small RNAs (Levenson and Sweatt, 2005; Barrett and Wood, 2008; Mikaelsson and Miller, 2011; Gräff and Tsai, 2013; Landry et al., 2013). Modifications in chromatin state influence the access of the transcriptional machinery to the genome. It is now well established that DNA methylation and histone acetylation are crucial epigenetic processes influencing long-term fear memory (Barrett and Wood, 2008; Mikaelsson and Miller, 2011; Gräff and Tsai, 2013). Histone deacetylase (HDAC) proteins deacetylate N-terminal lysine residues in histones, leading to a more compact chromatin structure and reduced gene transcription (Kouzarides, 2007).

HDAC inhibitors (HDACis) are the most widely investigated pharmacological agents modulating epigenetic processes. Administration of HDACis lead to increased acetylation and enhanced gene expression in neurons, resulting in a facilitation of synaptic plasticity as well as formation and extinction of fear conditioning (Levenson et al., 2004; Lattal et al., 2007; Vecsey et al., 2007; Bredy and Barad, 2008; Stafford et al., 2012). Acute systemic or intrahippocampal administration of HDACis enhances IA memory consolidation and rescues IA deficits related to aging or models of memory impairment (Silva et al., 2012; Blank et al., 2014, 2015, 2016; Sharma et al., 2015; Petry et al., 2016). Epigenetic alterations in the lateral amygdala, including increased histone H3 acetylation, are involved in the formation and reconsolidation of memory for auditory fear conditioning in rats. Intraamygdala infusion of the HDACi trichostatin A (TSA) enhances both the consolidation and reconsolidation of auditory fear memory (Maddox and Schafe, 2011; Monsey et al., 2011). Increased H3 acetylation in the amygdala is also related to accelerated extinction of auditory fear conditioning in mice after a systemic injection of an HDACi (Itzhak et al., 2012). However, previous studies have not examined the effects of HDAC inhibition in the amygdala on memory for IA. In the present study, we investigated the effects of TSA infused into the BLA at several time points after training, or immediately after retrieval, on the consolidation and extinction of IA memory in rats. Given the reported interactions between the BLA and dorsal hippocampus mentioned above, the enhancing effect of HDAC inhibitors on the expression of brain-derived neurotrophic factor (BDNF; Wu et al., 2008), and the role of hippocampal BDNF in promoting memory for IA (Chen et al., 2012), we also verified whether intra-BLA infusion of TSA resulted in an increase in hippocampal BDNF levels.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (220–350 g at time of surgery) were obtained from the institutional breeding facility (CREAL, ICBS, UFRGS) and maintained at the university hospital animal research facility (UEA, CPE-HCPA). Animals were housed four per cage in plastic cages with sawdust bedding and maintained on a 12 h light/dark cycle at a room temperature of 22 ± 2°C. The rats were allowed *ad libitum* access to standardized pellet food and water. All experiments took place during the light phase, between 8 AM and 5 PM.

Surgery

Rats were implanted under anesthesia with isoflurane (vaporized in 100% oxygen, at a dose of 5% for induction and 2% for maintenance, in a fraction of 0.5 l/min) with bilateral 14-mm, 23-gauge guide cannulae aimed 1.0 mm above the BLA, as described previously (Roesler et al., 2004; Jobim et al., 2012). Coordinates (anteroposterior, −2.8 mm from bregma; mediolateral, ±4.8 mm from bregma; ventral, −7.5 mm from skull surface) were obtained from the atlas of Paxinos and Watson (2007). Rats were allowed to recover at least 5 days after surgery before behavioral training.

Inhibitory Avoidance

Single-trial step-down IA was used as an established model of fear-motivated conditioning memory, where the animals learn to associate a location in the training apparatus (a grid floor) with an aversive stimulus (footshock). The general procedures for IA behavioral training and retention tests were described in previous reports (Jobim et al., 2012; Blank et al., 2014). The IA training apparatus was a 50 cm × 25 cm × 25 cm acrylic box (Albarsch, Porto Alegre, Brazil) with a floor composed of parallel caliber stainless steel bars (1 mm diameter) spaced 1 cm apart. A 7-cm wide, 2.5-cm high platform was placed on the floor of the box against one wall.

On training trials, rats were placed on the platform and their latency to step down on the grid with all four paws was measured with a digital chronometer. Immediately after stepping down on the grid, rats received a 0.4-mA, 3.0-s footshock and then removed from the apparatus immediately afterward. The first retention test trial was given 24 h after training by placing the rats on the platform and recording their latencies to step down. No footshock was presented during retention test trials. Step-down latencies on the retention test trial (maximum 300 s) were used as a measure of IA memory retention.

For IA extinction, rats were returned daily to the IA training context without footshock for 6 days as described previously (Roesler et al., 2014b; Petry et al., 2016). Rats that did not step down to the grid floor within 300 s during the first 24 h retention/extinction test trial were gently led by experimenter to the grid floor. Rats were given a 0.3 mA reminder footshock at the end of the fifth test, followed by an additional retention test 24 h later (Tronel and Alberini, 2007; Roesler et al., 2014b).

Drug Infusions

The general procedures for BLA infusions were described in previous reports (Jobim et al., 2012; Pedroso et al., 2013). At the time of infusion, a 27-gauge infusion needle was fitted into the guide cannula. The tip of the infusion needle protruded 1.0 mm beyond the guide cannula and was aimed at the BLA. Drug or vehicle was infused during a 30-s period. The infusion needle was left in place for an additional minute to allow diffusion of the drug away from the needle tip.

In the experiment to examine the memory consolidation, rats received a bilateral 0.5-μl infusion of TSA (Sigma-Aldrich, St. Louis, United States; 22 mM) dissolved in 50% ethanol in saline (vehicle, VEH; Vecsey et al., 2007) into the BLA at different times after IA training. Different groups of rats were used for each infusion time point. Control animals received VEH in the same condition. In the memory extinction experiment, rats received a bilateral 0.5-μl infusion of TSA (22 mM) or VEH immediately after the first test trial. The TSA dose was chosen on the basis of previous findings from our group showing that it enhanced IA memory consolidation when given into the dorsal hippocampus (Blank et al., 2014). Drug solutions were prepared freshly before each experiment.

Measurement of BDNF Levels

A separate group of rats was given one IA training trial as described above, followed immediately by an intra-BLA infusion of TSA (22 mM) or VEH. Four hours later, the rats were sacrificed by decapitation, their brains were removed and the BLA and hippocampus were quickly dissected out, immediately snap-frozen in liquid nitrogen and stored at −80°C until BDNF measurement. The posttraining time for BDNF measurement was chosen on the basis of a previous study showing that hippocampal BDNF levels increased 4 h after learning (Goulart et al., 2010). BLA and hippocampal BDNF was measured as described previously (Kauer-Sant'Anna et al., 2007; Goulart et al., 2010), using sandwich enzyme-linked immunosorbent assay (ELISA) commercial kits according to the manufacturer's instructions (ChemiKine™, CYT306, Merck Millipore, Temecula, United States). Briefly, samples were homogenized in phosphate-buffered solution with 1 mM phenylmethylsulfonyl fluoride and 1 mM ethyleneglycoltetraacetic acid. Microtiter plates (96-well flat-bottom) were coated for 24 h with the samples diluted 1:2 in sample diluents and the standard curve ranged from 7.8 to 500 pg/ml of BDNF. The plates were then washed four times with wash buffer and a monoclonal anti-BDNF rabbit antibody (1:1000) was added to each well and incubated for 3 h at room temperature. After washing, a peroxidase-conjugated anti-rabbit antibody (horseradish peroxidase enzyme; 1:1000) was added to each well and incubated for 1 h at room temperature. After addition of streptavidin enzyme, substrate (3,3′,5,5′-tetramethylbenzidine) and stop solution, the amount of BDNF was determined by absorbance at 450 nm in a spectrophotometer. Total protein was measured using the Bradford's method with bovine serum albumin as the standard.

Histology

A 0.5-μl infusion of a 4% methylene blue solution was infused into the cannulae 24–48 h after the end of behavioral testing. Rats were killed by decapitation 15 min later, and their brains were removed and stored in 10% formalin for at least 72 h. At least 24 h before sectioning, brains were placed in a 20% sucrose solution in water for cryoprotection. Coronal sections of 50 μm were cut on a cryostat, mounted on gelatin-coated slides, stained with hematoxylin and eosin and examined under light microscopy. The extension of the methylene blue dye was taken as indicative of diffusion of the drugs previously given each rat (Roesler et al., 2004; Jobim et al., 2012). Rats with incorrect cannula placements were excluded from the statistical analyses.

Statistics

Non-parametric tests were used to analyze retention test latencies because of the 300-s cut-off imposed on retention test trials. Training and retention test step-down latencies were analyzed using a Kruskal–Wallis test followed by two-tailed Mann–Whitney *U*-tests. Student's *t*-tests for independent samples were used for comparisons of BDNF levels between controls and TSA-treated groups within each brain area (BLA or hippocampus). In all comparisons, $p < 0.05$ was considered to indicate statistical significance. All data are shown as mean ± standard error of mean (SEM).

RESULTS

Time Course of Consolidation Enhancement by Intra-BLA Administration of TSA

The first set of experiments examined the effects of intra-BLA administration of TSA at different posttraining intervals on IA memory consolidation. Rats were given IA training followed by a bilateral infusion of VEH or TSA (22 mM) into the BLA immediately (VEH, $N = 10$; TSA, $N = 9$), 1.5 h (VEH, $N = 13$; TSA, $N = 13$), 3 h (VEH, $N = 14$; TSA, $N = 14$), or 6 h (VEH, $N = 13$; TSA, $N = 13$) after training. All rats were tested for retention 24 h later after training.

Results are shown in **Figure 1**. Infusions given immediately after training had no effect ($p = 0.43$, **Figure 1A**). However, retention test latencies were significantly higher compared to VEH-treated controls in rats infused with TSA at 1.5 ($p < 0.01$, **Figure 1B**), 3 ($p < 0.05$, **Figure 1C**), and 6 ($p < 0.01$, **Figure 1D**) h posttraining. There were no significant differences between groups in training trial latencies (immediate posttraining infusions, $p = 0.10$; 1.5 h posttraining infusions, $p = 0.25$; 3 h posttraining infusions, $p = 0.96$; 6 h posttraining infusions, $p = 0.09$).

Intra-BLA Administration of TSA Delays IA Memory Extinction

We then went on to verify whether TSA into the amygdala would affect IA extinction. Rats were given bilateral intra-BLA infusions

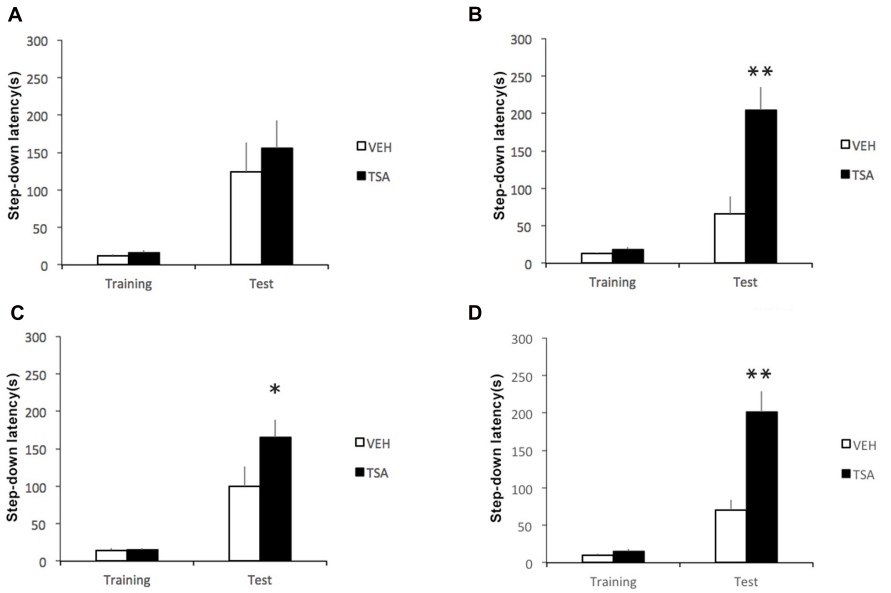

FIGURE 1 | Enhancement of later phases of IA memory consolidation by HDAC inhibition in the amygdala shortly after learning. Rats were given a single IA training trial followed by an infusion of VEH or TSA (22 mM) into the BLA **(A)** immediately, **(B)** 1.5 h, **(C)** 3 h, or **(D)** 6 h after training. Retention was tested 1 day after training in all groups. Data are mean ± SEM latencies to step down; N = 9–14 rats per group; $*p < 0.05$, $**p < 0.01$ compared to respective controls.

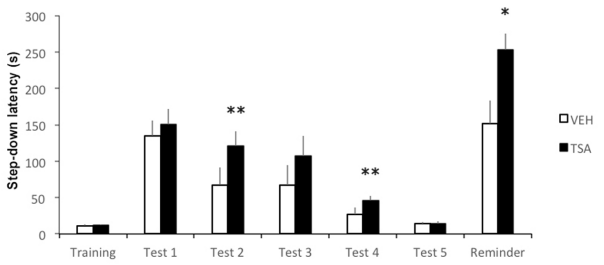

FIGURE 2 | Amygdalar HDAC inhibition delays IA memory extinction. Rats were given a single IA training trial and tested for retention 24 h later (Test 1). This first test trial also served as extinction training. An infusion of VEH or TSA (22 mM) was given into the BLA immediately after Test 1. Retention was tested once daily from days 1 to 5 after Test 1 (Tests 2–5). A mild reminder footshock was given after Test 5 and rats were tested again 1 day later (Reminder). Data are mean ± SEM latencies to step down; VEH, $N = 11$, TSA, $N = 12$; $*p < 0.05$; $**p < 0.01$ compared to controls.

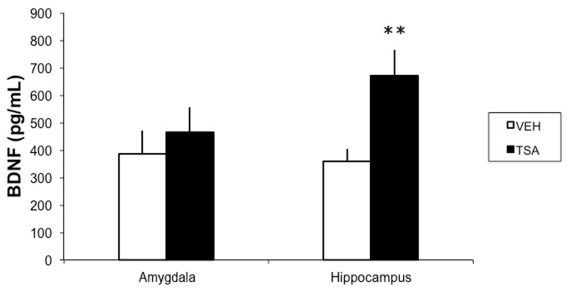

FIGURE 3 | HDAC inhibition in the BLA results in increased BDNF levels in the dorsal hippocampus in rats given IA training. Rats were given a bilateral intra-BLA infusion of VEH or TSA (22 mM) immediately after IA training. Four hours later, they were sacrificed and the BLA and dorsal hippocampus levels were removed for BDNF measured with an ELISA. Data are mean ± SEM pg of BDNF/ml of protein; VEH, N = 12, TSA, N = 10; $**p < 0.01$ compared to respective controls.

of VEH ($N = 11$) or TSA ($N = 12$) immediately after the first test trial (Test 1), which served as an extinction training trial. All rats were tested for extinction 1 (Test 2), 2 (Test 3), 3 (Test 4), and 4 (Test 5) days after Test 1. Immediately after Test 5, rats were given a reminder footshock and retention was tested again 1 day later in the absence of footshock.

Results are shown in **Figure 2**. Latencies were significantly higher in TSA-treated rats compared to controls in Test 2 ($p < 0.01$) and Test 4 ($p < 0.01$). The difference in Test 3 latencies did not reach significance ($p = 0.10$), in spite of the apparently higher latency in TSA-treated rats. Both groups reached similar levels of extinction by Test 5 ($p = 0.68$). Overall, the results indicate that TSA delayed extinction. Rats treated with TSA also showed significantly higher latencies than VEH controls after being presented with a reminder footshock ($p < 0.05$), supporting the possibility that the fear response in TSA-treated rats was more resistant to extinction. There were no significant differences between groups in latencies during training ($p = 0.68$).

TSA Infusion into the BLA Increases BDNF Levels in the Hippocampus But Not Amygdala in IA-Trained Rats

In a separate group of rats given IA training followed by an intra-BLA infusion of VEH ($N = 12$) or TSA ($N = 10$)

FIGURE 4 | Infusion placements into the BLA. Representative photomicrograph illustrating placement of a cannula and needle tip, and schematic diagram of a coronal section of the rat brain (anteroposterior, –2.8 mm from bregma), adapted from the atlas of Paxinos and Watson (2007), depicting the diffusion of methylene blue in the BLA for rats included in the statistical analysis.

immediately afterward, and sacrificed for BDNF measurements 4 h later, intra-BLA TSA induced a significant increase in BDNF levels in the hippocampus ($p < 0.01$), but not in the BLA ($p = 0.85$; **Figure 3**).

Histology

All animals (144 rats) included in the final analysis of IA had cannula bilaterally placed in the BLA. **Figure 4** shows a representative photomicrograph illustrating placement of a cannula and needle tip, as well as a schematic drawing of the diffusion of methylene blue, which indicates infusion placements and spread of drug infusions within the BLA.

DISCUSSION

Previous studies have shown that administration of HDACis into the amygdala around the time of training or retrieval resulted in enhanced consolidation and reconsolidation of memory for fear conditioning (Maddox and Schafe, 2011; Monsey et al., 2011), and systemic injections of HDACi could also accelerate fear extinction (Lattal et al., 2007; Bredy and Barad, 2008; Itzhak et al., 2012; Stafford et al., 2012). The present study reveals several novel aspects related to the amygdalar HDAC involvement in fear memory. First, we provide the first report of the effects of HDAC inhibition in the amygdala on memory of IA. Second, we show that HDACis can be more effective in enhancing memory when given at later time points during consolidation (up to at least 6 h after training) than when administered before or shortly after learning. Third, we show that HDAC inhibition can impair rather than facilitate fear extinction. Finally, we provide the first evidence that inhibiting HDAC within the amygdala can result in an increase in BDNF levels in the dorsal hippocampus.

In contrast to previous reports (Lattal et al., 2007; Bredy and Barad, 2008; Itzhak et al., 2012; Stafford et al., 2012), we found that treatment with an HDACi delayed rather than facilitated fear extinction. In IA, memory reactivation at the time of testing can initiate either one of two competing processes: further memory strengthening, likely mediated by reconsolidation; or memory extinction (Vianna et al., 2001; Jobim et al., 2012; Pedroso et al., 2013; Furini et al., 2014; Roesler et al., 2014b). It is possible that intra-BLA TSA given after retrieval acts by enhancing reconsolidation to make the original memory more resistant to extinction. The possibility that the original memory for training was stronger in TSA-treated rats is supported by the finding that, compared to controls, they showed an increased avoidance response after exposure to a reminder shock.

Perhaps the most intriguing finding of the present report was that intra-BLA TSA administration led to an increase in BDNF protein content in the dorsal hippocampus, but not in the amygdala. It is well established that BDNF, which acts by activating its receptor, TrkB, resulting in the stimulation of a range of intracellular kinase signaling pathways including phospholipase C/protein kinase C, extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase, plays a major role in synaptic plasticity and memory formation (Huang and Reichardt, 2003; Minichiello, 2009; Yoshii and Constantine-Paton, 2010). Consolidation of IA memory requires BDNF/TrkB signaling that accompanies protein synthesis in the dorsal hippocampus (Bambah-Mukku et al., 2014), and intrahippocampal administration of an anti-BDNF antibody before training impairs IA retention (Chen et al., 2012). Gene transcription for BDNF is stimulated by HDACis (Wu et al., 2008; Koppel and Timmusk, 2013), and systemic HDACi treatment increases protein levels of BDNF in the rat brain (Kim et al., 2009). In addition, administration of an HDACi into the hippocampus rescues the impairment of IA memory consolidation produced by TrkB inhibition (Blank et al., 2016).

Thus, promoting BDNF expression in the hippocampus could be a crucial mechanism enabling amygdalar HDAC inhibition to enhance different phases of IA memory consolidation. However, TSA infused into the BLA immediately after training, which resulted in an increase in hippocampal BDNF, did not affect retention. It is possible that the increase in BDNF caused by posttraining TSA was related to resistance to extinction, although that BDNF has been shown to induce fear extinction under some circumstances (Peters et al., 2010). Also, what could be the mechanism mediating the increase in hippocampal BDNF after inhibition of amygdalar HDAC? Previous studies have indicated that BLA activity influences gene expression related to IA memory formation in the hippocampus and related brain areas. For instance, BLA activity is required to enable the effects of memory-enhancing agents, including HDACis, given into the hippocampus or entorhinal cortex (Roozendaal and McGaugh, 1997; Roozendaal et al., 1999; Roesler et al., 2002; Blank et al., 2014). Importantly, intra-BLA infusion of a memory-enhancing drug, the beta-adrenoreceptor agonist clenbuterol, resulted in an increase in dorsal hippocampal levels of activity-regulated cytoskeletal protein (Arc, also called Arg 3.1), an immediate-early gene involved in synaptic plasticity and memory consolidation, whereas BLA inactivation by a lidocaine infusion decreased Arc content in the hippocampus (McIntyre et al., 2005; McReynolds et al., 2014). Noradrenaline can enhance histone acetylation (Maity et al., 2016), and possible mechanisms mediating BDNF influences on synaptic plasticity include an upregulation of Arc levels (Yin et al., 2002; Ying et al., 2002). Therefore, the possibility that increased histone acetylation in the BLA can enhance hippocampal BDNF expression is consistent with previously described neurotransmitter and gene expression

pathways involved in BLA-hippocampal interactions during memory formation.

Formation of IA memory has been previously shown to involve ERK/MAPK signaling in the hippocampus and BLA 3 h posttraining, but not shortly after training (Walz et al., 2000). Increases in histone H3 acetylation in the amygdala induced by fear conditioning are downstream of ERK/MAPK signaling (Monsey et al., 2011), and BDNF mediates enhancing effects on memory through MAPK activation (Revest et al., 2014). Therefore, stimulation of hippocampal MAPK activity through up-regulation of BDNF induced by intra-BLA TSA arises as another candidate mechanism for the effects observed in our study. It is likely that the enhancing effect of intra-BLA TSA also involves the combined action of several other mechanisms. TSA is a hydroxamic acid containing a functional group that interacts with the critical zinc atom at the base of the catalytic pocket of HDACs, thus inhibiting their activity. In addition to inhibiting class I HDACs, which are localized predominantly to the cell nucleus, TSA also inhibits HDAC6, the main cellular cytoplasmic deacetylase (Bhalla, 2005; Bolden et al., 2006). Moreover, HDACis might display extra-epigenetic effects, such as direct interactions with cytoplasmic cell signaling pathways and acetylation of non-histone proteins (Chen et al., 2005; Glozak et al., 2005).

In summary, the present findings reveal novel aspects of the involvement of amygdalar epigenetic mechanisms in fear memory, by showing that HDAC inhibition in the amygdala can enhance a later phase of consolidation, delay extinction, and possibly act by increasing BDNF levels in the dorsal hippocampus. Our findings raise the exciting possibility that epigenetic manipulations within the BLA affect memory processes by influencing the expression of molecules mediating synaptic plasticity in the hippocampus rather than the amygdala itself.

ETHICS STATEMENT

All experimental procedures were performed in accordance with the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI) and approved by the institutional Animal Care Committee (CEUA-HCPA) under protocol number 140429.

AUTHOR CONTRIBUTIONS

FV, GS, NS, CdF, and RR designed the research; FV, MV, ML, FP, and CdF performed experiments; FV analyzed the data; FV, RdA, GS, NS, and RR interpreted and discussed the data; GS, RdA, NS, and RR provided materials; FV and RR wrote the paper; FV, MV, ML, FP, RdA, GS, NS, CdF, and RR revised the manuscript.

FUNDING

This research was supported by the National Council for Scientific and Technological Development (CNPq; grant numbers 484185/2012-8 and 303276/2013-4 to RR); Coordination for the Improvement of Higher Education Personnel (CAPES); the HCPA institutional research fund (FIPE/HCPA; number 140429); the South American Office for Anticancer Drug Development; and the Children's Cancer Institute (ICI).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 14 November 2017
doi: 10.3389/fphar.2017.00822

CaMKII Requirement for *in Vivo* Insular Cortex LTP Maintenance and CTA Memory Persistence

*Yectivani Juárez-Muñoz, Laura E. Ramos-Languren and Martha L. Escobar**

División de Investigación y Estudios de Posgrado, Facultad de Psicología, Universidad Nacional Autónoma de Mexico, Mexico City, Mexico

Calcium-calmodulin/dependent protein kinase II (CaMKII) plays an essential role in LTP induction, but since it has the capacity to remain persistently activated even after the decay of external stimuli it has been proposed that it can also be necessary for LTP maintenance and therefore for memory persistence. It has been shown that basolateral amygdaloid nucleus (Bla) stimulation induces long-term potentiation (LTP) in the insular cortex (IC), a neocortical region implicated in the acquisition and retention of conditioned taste aversion (CTA). Our previous studies have demonstrated that induction of LTP in the Bla-IC pathway before CTA training increased the retention of this task. Although it is known that IC-LTP induction and CTA consolidation share similar molecular mechanisms, little is known about the molecular actors that underlie their maintenance. The purpose of the present study was to evaluate the role of CaMKII in the maintenance of *in vivo* Bla-IC LTP as well as in the persistence of CTA long-term memory (LTM). Our results show that acute microinfusion of myr-CaMKIINtide, a selective inhibitor of CaMKII, in the IC of adult rats during the late-phase of *in vivo* Bla-IC LTP blocked its maintenance. Moreover, the intracortical inhibition of CaMKII 24 h after CTA acquisition impairs CTA-LTM persistence. Together these results indicate that CaMKII is a central key component for the maintenance of neocortical synaptic plasticity as well as for persistence of CTA-LTM.

Keywords: CaMKII, CTA, neocortical-LTP, memory persistence, insular cortex

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Peter K. Giese, King's College London, United Kingdom
Jorge Medina, Universidad de Buenos Aires, Argentina
Christine Gall, University of California, Irvine, United States

***Correspondence:**
Martha L. Escobar
mescobar@unam.mx

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *29 June 2017*
Accepted: *30 October 2017*
Published: *14 November 2017*

Citation:
Juárez-Muñoz Y, Ramos-Languren LE and Escobar ML (2017) CaMKII Requirement for in Vivo Insular Cortex LTP Maintenance and CTA Memory Persistence. Front. Pharmacol. 8:822. doi: 10.3389/fphar.2017.00822

INTRODUCTION

Learning and memory rely on long-lasting changes in synaptic efficiency within neural networks. Long-term potentiation (LTP) is a long-lasting and activity-dependent enhancement of synaptic strength that is widely expressed across the brain (Malenka and Bear, 2004; Rodríguez-Durán et al., 2011). Studies in the neocortex and hippocampus have demonstrated that training in several learning tasks drive modifications of synaptic strength (Rioult-Pedotti et al., 2000; Whitlock et al., 2006; Cooke and Bear, 2010; Liu et al., 2017; Rodríguez-Durán et al., 2017). The insular cortex (IC) is a region of the temporal neocortex implicated in the acquisition and storage of conditioned taste aversion (CTA). CTA is a well-established learning and memory paradigm in which an animal acquires aversion to a novel taste when it is associated with nausea (Bernstein and Koh, 2007; Bermúdez-Rattoni, 2014; Rivera-Olvera et al., 2016). Previous studies demonstrated that high frequency stimulation of the basolateral amygdaloid nucleus (Bla) elicits LTP in the IC (Escobar et al., 1998a,b; Jones et al., 1999). Moreover, we have shown that induction of LTP in the Bla-IC pathway before CTA training enhances the retention of this task (Escobar and Bermúdez-Rattoni, 2000; Castillo et al., 2006).

Research on the cellular basis of learning and memory has identified some key molecules involved in the processes of acquisition and consolidation of information (Lamprecht and LeDoux, 2004). However, little is known about the processes involved in the permanence of long-term memory (LTM). Recently, the search of molecular mechanisms involved in LTM storage has highlighted a significant participation of calcium/calmodulin dependent protein kinase II (CaMKII). The increase in calcium concentration after LTP induction leads to CaMKII autophosphorylation, an event that makes CaMKII activity persist even after the decay of calcium concentration (Lisman et al., 2002, 2012; Colbran and Brown, 2004). However, it has been demonstrated that this autonomous activity is only transient (Lengyel et al., 2004; Otmakhov et al., 2015; Murakoshi et al., 2017), while Thr286 phosphorylation has been proved as a persistent event, that has been observed up to 8 h after stimulation (Ahmed and Frey, 2005). In this regard, it has been observed that synaptic potentiation in hippocampal CA1 region is reverted when ant-CaMKIINtide, a noncompetitive inhibitor of CaMKII, is applied during the maintenance phase of CA1-LTP *in vitro* (Sanhueza et al., 2007). Similar results have been observed by our research group when CaMKIINtide is infused in CA3 region during the maintenance phase of *in vivo* mossy fiber (MF)-LTP (Juárez-Muñoz et al., 2017). In a recent study it has been proved that expression of a transient dominant-negative form of CaMKII erases a previously stablished hippocampal-dependent memory, pointing to a role of this molecule for stable memory storage (Rossetti et al., 2017). It has also been shown that training in a spatial task elicits increments in hippocampal CaMKII autophosphorylation (Tan and Liang, 1996). Furthermore, intrahipocampal pharmacogenetic inhibition of CaMKII activity impairs retention of spatial memory (Babcock et al., 2005). Importantly, it has been shown that although mice heterozygous for a CaMKII null mutation have normal memory retention for contextual fear and water maze tasks 1–3 days after training, these animals exhibit amnesia when tested 10–50 days post-training (Frankland et al., 2001), revealing a role for CaMKII in the persistence of memory.

Since little is known about the molecular actors implicated in the maintenance of synaptic plasticity and LTM, in the present work we evaluated the role of CaMKII in the maintenance of *in vivo* Bla-IC LTP as well as in the persistence of CTA-LTM.

MATERIALS AND METHODS

Animals

Seventy-three male Wistar rats, weighing 360–390 g were prepared for the present study. Rats were individually caged and maintained on a 12:12 light–dark cycle at 22° C with water and food available *ad libitum* except where indicated (Martínez-Moreno et al., 2016). Experiments were performed in accordance with the Norma Oficial Mexicana and with the approval of the Animal care committee of the Faculty of Psychology of the National Autonomous University of Mexico.

Electrophysiology Procedure

Electrophysiological recordings were performed in anesthetized rats as previously described (Escobar et al., 1998a; Rodríguez-Durán et al., 2011; Rivera-Olvera et al., 2016). Briefly, rats were anesthetized with pentobarbital (50 mg/kg i.p.). Responses were recorded by using a monopolar microinfusion electrode placed in the IC. Constant current stimulation (50–70 μA monophasic pulses, 0.25 ms duration) was applied to the Bla unilaterally using a stainless steel bipolar electrode. The microinfusion electrodes were coupled to 10 μl Hamilton syringes (Reno, NV, USA) driven by a microinfusion pump (Cole Parmer Co., Vernon Hills, IL, USA). Evoked responses from IC were measured by recording the EPSP slope. During the 30 min baseline period responses were evoked at 0.05 Hz. LTP was induced by delivering 10 trains of 100 Hz/1 s with an intertrain interval of 20 s. Animals with unclear electrode placement were discarded.

Western Blot

Rats were decapitated and the ipsilateral recorded IC area was microdissected. The tissues were subsequently sonicated into a lysis buffer (50 mM Tris-HCl pH 6.8, 20 mM NaCl, 2 mM EDTA, 10% glycerol, 10% triton) supplemented with 10 mM protease inhibitors (Mini Complete, Roche, Manheim, Germany); as well as with phosphatase inhibitors (50 mM NaF, 4 mM Na3VO4, 10 mM NaPPi). Following sonication, samples were centrifuged at 14,000 rpm for 20 min at 4°C and the supernatant was obtained. Protein concentration was measured using Bradford assay, with bovine serum albumin as standard. An equivalent amount of protein for each sample was resolved in 12% SDS-acrylamide gels; blotted electrophoretically and blocked 90 min in TBST buffer (Tris buffered saline containing 0.01%, Tween-20, pH 7.4) containing 5% non-fat milk (Castillo and Escobar, 2011). Membranes were incubated overnight at 4°C with anti-phospho CaMKII T286/287 antibody (1:1,000, #06-881, Millipore, Darmstadt, Germany) for the detection of phosphorylated form of CaMKII and with anti-CaMKII antibody (1:500, #5306, Santa Cruz, CA, USA) for CaMKII total. The phosphorylated isoforms were normalized to the total isoform as a ratio, which was presented as a percentage value in histograms. We performed densitometry using the software off-line ImageJ (NIH, USA).

Cannulae Implantation

Using a previously described procedure, animals were bilaterally implanted in the IC with stainless steel guide cannulae (Moguel-González et al., 2008; Rodríguez-Serrano et al., 2014). Microinjectors were attached by polyethylene tubing to a 10-μl Hamilton syringe driven by a microinfusion pump (Cole Parmer Co., Vernon Hills, IL, USA). Animals were allowed to recover for 1 week after surgery. Histological analysis was performed on all groups to verify the injector tip location.

CTA

As previously described (Rivera-Olvera et al., 2016), 7 days after surgery animals were trained to drink water twice a day from a graduated cylinder, during 10 min trials for 3 days. On the acquisition session, water was replaced by a saccharin solution

0.1% (Sigma, St. Louis, MO), and 10 min later, animals were intraperitoneally injected with LiCl (0.15 M; 7.5 ml/kg). During the aversion test 0.1% saccharin solution was presented again after two more days of baseline consumption. The strength of aversion was measured through the reduction of saccharin consumption.

Experimental Design

To analyze the effect of CaMKII inhibition on the maintenance of Bla-IC-LTP, animals were divided into the following treatment groups: (1) HFS group ($n = 7$), which underwent surgery, had electrodes implanted and received high frequency stimulation (HFS) capable of inducing LTP; (2) HFS+CaMKIINtide group ($n = 7$), which in the same conditions of HFS group received intracortical microinfusion of myr-CaMKIINtide (5 μM/μl ACSF/.02 μl/min; Sanhueza et al., 2007; Juárez-Muñoz et al., 2017) prepared with artificial cerebrospinal fluid (ACSF) as vehicle 2 h after HFS delivery; (3) HFS+ACSF group ($n = 7$) which under the same conditions as the HFS+CaMKIINtide group, received intracortical microinfusion of ACSF (1 μl); (4) CaMKIINtide group ($n = 7$) which had electrodes implanted and without prior manipulation received intracortical microinfusion of myr-CaMKIINtide (5 μM/μl ACSF/0.02 μl/min). In order to analyze the state of phosphorylation of CaMKII in the presence of myrCaMKIINtide, three additional animals from each of HFS+ACSF and HFS+CaMKIINtide groups were decapitated and tissue was obtained 130 min after HFS application (i.e., 10 min after myr-CaMKIINtide administration) (**Figure 1A**).

To evaluate the effect of CaMKII inhibition on the maintenance of CTA-LTM, animals were divided into the following treatment groups: (1) CTA+STM+CaMKIINtide group ($n = 10$), which was trained in CTA and was subjected to a short-term memory aversion test (STM) carried out 4.5 h after the acquisition in order to corroborate the association of saccharin with gastric malaise. One day after acquisition rats received bilateral intracortical microinfusion of myr-CaMKIINtide (5 μM/μl ACSF/0.02 μl/min; Sanhueza et al., 2007; Juárez-Muñoz et al., 2017) prepared with artificial cerebrospinal fluid (ACSF). LTM was assessed 48 h after infusion; (2) CTA+STM+ACSF ($n = 10$), which under the same conditions of CTA+STM+CaMKIINtide group received bilateral intracortical microinfusion of ACSF (1 μl); (3) CTA+CaMKIINtide ($n = 10$), which was trained in CTA and received bilateral intracortical microinfusion of myr-CaMKIINtide in absence of STM aversion test in order to prevent the inherent interference to retrieval process (Nader and Einarsson, 2010); (4) pCTA+STM+CaMKIINtide ($n = 9$), which was pseudotrained in CTA (during the conditioning session PBS was delivered instead of LiCl) and received bilateral intracortical microinfusion of myr-CaMKIINtide (**Figure 2A**).

RESULTS

Histology

Histological examinations revealed that the stimulating and recording electrodes were correctly located in the Bla and the IC, respectively, in all animals included in the present analysis (**Figure 1B**). Similarly, injectors were correctly placed in the IC for all groups (**Figure 2B**).

Electrophysiology

The IC EPSP consisted of potentials of 0.47 ± 0.004 mV (mean ± SEM), elicited with 50–70 μA current pulses of 0.1–0.25 ms duration. These responses initiate at 2–3 ms post-stimulation and presented their peak at 7–9 ms with an average slope of 0.019 ± 0.003 (mean ± SEM), in agreement with previous studies (Escobar et al., 1998a, 2002; Jones et al., 1999; Rodríguez-Durán et al., 2011).

CaMKII Is Necessary for the Maintenance of *in Vivo* HFS Induced Potentiation of Synaptic Transmission at Bla-IC Pathway of Adult Rats

As previously described, HFS produced an enhancement in the IC field EPSP slope values, with a duration of at least 3 h (HFS group). The application of myr-CaMKIINtide (5 μg/1 μl) in the IC (HFS+CaMKIINtide group) blocked the maintenance phase of synaptic potentiation when the inhibitor was applied 2 h after HFS (**Figure 1C**), while infusion of ACSF (1 μl, HFS+ACSF group) had no effect over the maintenance phase of LTP. Remarkably, the application of the inhibitor in the absence of HFS did not have any effect over baseline transmission (CaMKIINtide group). ANOVA analysis for slope increases revealed highly significant group differences [$F_{(3, 24)} = 71.28$; $P < 0.001$]. *Post-hoc* analysis with Fisher's test showed significant differences between the HFS+CaMKIINtide group and all the groups that received HFS ($P < 0.001$). At 1 h post-infusion, the percent changes (±SEM) in the EPSP slope for the HFS, HFS+CaMKIINtide, HFS+ACSF and CAMKIINtide groups were 128.96 ± 4.58, 94.89 ± 7.22, 125.76 ± 4.18, and 95.59 ± 6.37 respectively.

Western blot analysis showed that phosphorylation of the two main cortical isoforms of CaMKII (α and β) were decreased by myr-CaMKIINtide administration. As shown in **Figure 1D**, the administration of the inhibitor of CaMKII during the maintenance phase of synaptic potentiation decreased CaMKII phosphorylation in the IC region of animals from group HFS+CaMKIINtide, as compared to HFS+ACSF group [Two-Way ANOVA; $F_{(1,8)} = 52.00$; $p < 0.05$]. No changes were observed in CaMKII total expression.

CamkII Inhibition Impairs the Maintenance of CTA-LTM

No significant differences were found among groups neither in the baseline water intake nor during the acquisition session. Intracortical microinfusion of myr-CaMKIINtide 24 h after CTA acquisition lead to a memory impairment during LTM aversion test, performed 48 h after the inhibitor microinfusion, i.e., 72 h after the CTA acquisition. Two-way ANOVA revealed significant group differences [$F_{(3, 35)} = 22.75$, $p < 0.001$]. *Post-hoc* analysis with Fisher's test revealed that groups CTA+STM+CaMKIINtide and CTA+CaMKIINtide showed significant differences compared to CTA+STM+ACSF and

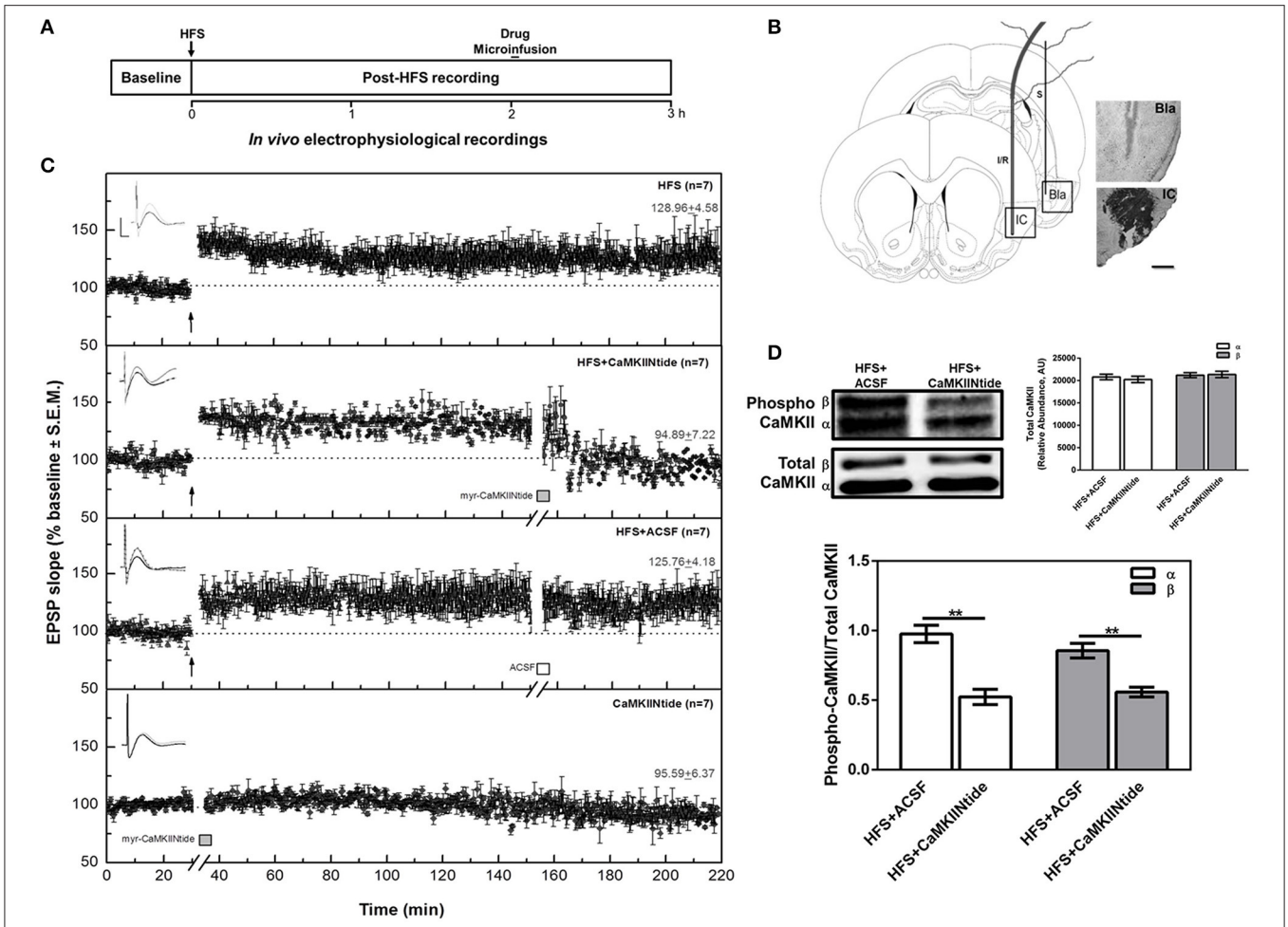

FIGURE 1 | CaMKII inhibition reverts IC-LTP. **(A)** Schematic representation of the experimental procedure. **(B)** Schematic representation and representative micrographs of electrode placement showing the stimulated (S) and infused/recorded (I/R) sites in a coronal plane. The representative microphotography of IC shows the CaMKIINtide diffusion through the combination of the drug with Ponceau red. Bla, basolateral amygdaloid nucleus; IC, insular cortex. Scale bar: 1 mm. **(C)** Plot of IC evoked responses from HFS, HFS+CaMKIINtide, HFS+ACSF and CaMKIINtide groups. Note that the inhibition of CaMKII reverts Bla-IC potentiation. The top of each graph shows representative examples of IC field potentials obtained before (black line), 120 min after HFS (gray line) and 60 min after the infusion (dotted line). Scale bar: 5 ms, 0.5 mV. Bars indicate the infusion period (5 min). Arrows indicate HFS delivery. **(D)** Representative Western blots and densitometric analysis of phosphorylated and total CaMKII from HFS+CaMKIINtide and HFS+ACSF groups. Note that infusion of the inhibitor during IC-LTP maintenance phase decreased phosphorylation of CaMKII. AU, Arbitrary units. $^{**}p < 0.01$.

pCTA+STM+CaMKIINtide groups ($p < 0.001$). During the STM aversion test groups CTA+STM+VEH and CTA+STM+CaMKIINtide showed a significant reduction in the consumption of saccharin solution, confirming that the association with the gastric malaise was well established, as shown in **Figure 2C**.

DISCUSSION

Relatively little is known about the LTP maintenance processes that underlie the persistence of synaptic memory. The results of the present study demonstrate that CaMKII is necessary for the maintenance of *in vivo* HFS induced potentiation of synaptic transmission at Bla-IC pathway, considered as an important excitatory circuit implicated in the acquisition and storage of CTA. The intracortical application of the CaMKII inhibitor, myr-CaMKIINtide, 2 h after HFS delivery suppressed the maintenance phase of synaptic potentiation. The suppression can most likely be attributed to a decrease in the CaMKII phosphorylation, as confirmed by the immunoblot analysis. Remarkably, the application of the inhibitor in the absence of HFS did not have any effect over baseline transmission. Previous studies have revealed an important potential role for CaMKII in memory maintenance but most have been performed in the hippocampus. In this regard, Sanhueza and colleagues, have reported that application of an inhibitor of CaMKII lead to the decrement of synaptic potentiation during the maintenance phase of *in vitro* N-methyl-D-aspartate (NMDA) receptors dependent LTP in CA1 region (Sanhueza et al., 2007, 2011),

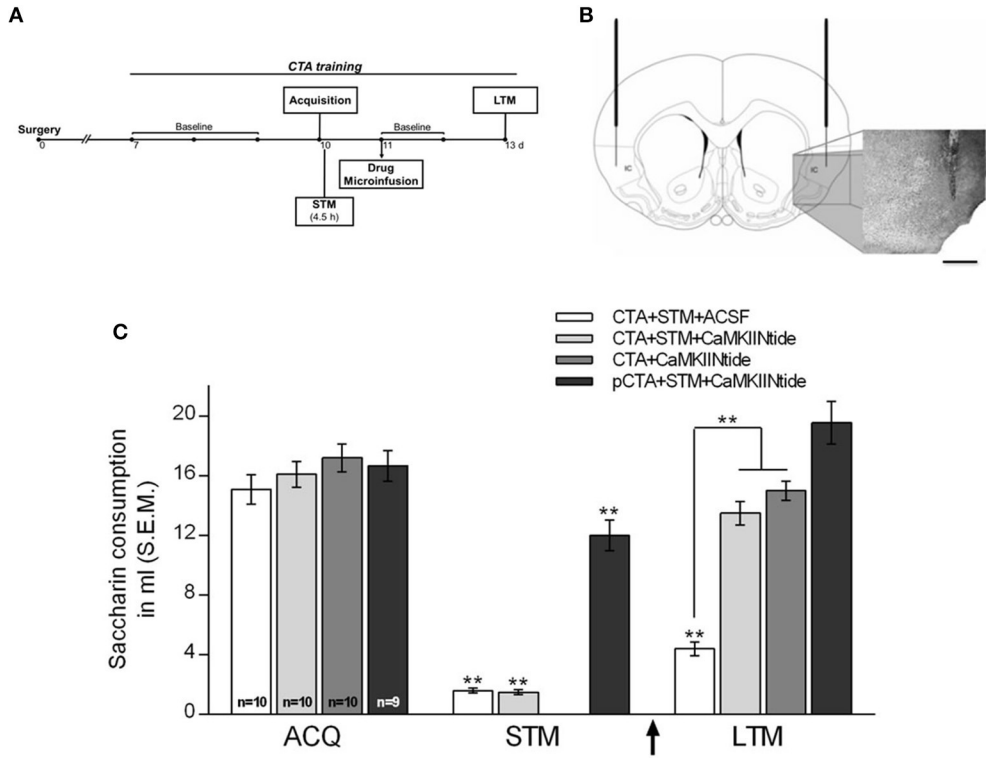

FIGURE 2 | CaMKII inhibition impairs the maintenance of CTA-LTM. **(A)** Schematic representation of the experimental procedure. **(B)** Schematic representation and representative micrographs showing cannulae placement. IC, insular cortex. Scale bar: 1 mm. **(C)** Saccharin consumption during acquisition, short-term memory aversion test (STM) and long-term memory aversion test (LTM) for CTA+STM+ACSF, CTA+STM+CaMKIINtide, CTA+CaMKIINtide and pCTA+STM+CaMKIINtide groups. Note that local infusion of myr-CaMKIINtide in the IC 24 h after acquisition, either in the presence or absence of STM-test produced a severe deficit during the LTM aversion test. $^{**}p < 0.001$. Arrow indicates microinfusion.

and recently our research group has observed similar results in the hippocampal MF pathway *in vivo* (Juárez-Muñoz et al., 2017).

Regarding the mechanisms, it has been described that autophosphorylation of CaMKII generates autonomous activity, as well as post-synaptic translocation to interact with target proteins, such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptors (Strack and Colbran, 1998; Baucum et al., 2015). Indeed, the absence of T286 CaMKII autophosphorylation blocks the hippocampal NMDA dependent LTP (Giese et al., 1998; Yamagata et al., 2009). In addition, it has been well established that autonomous CaMKII phosphorylates AMPA receptors, an event that accounts for LTP induction (Derkach et al., 1999; Ghosh et al., 2015). On the other hand, it has been proposed that the association of CaMKII with GluN2B subunits of NMDA receptors is a central mechanism required for the maintenance phase of LTP in the hippocampus (Sanhueza et al., 2011). CaMKII-GluN2B binding persists even after LTP stimulus decay and this association leaves the kinase in a partially autonomous conformation that mediates its redistribution (Strack et al., 2000; Bayer et al., 2006; O'Leary et al., 2011). Accumulation of CaMKII-GluN2B at the post-synaptic density promotes the capture of multiple proteins including actinin, densin, delta-catenin and N-cadherin, acting as a structural scaffold for a series of binding reactions that together contribute to LTP maintenance (Sanhueza and Lisman, 2013). Moreover, it is known that structural reorganization as a result of activity is required for maintenance of synaptic strength (Lamprecht and LeDoux, 2004; Lynch et al., 2007). In this regard, it has been demonstrated that CaMKII inhibition during the maintenance phase of MF-LTP prevents the activity-dependent morphological reorganization in this pathway (Juárez-Muñoz et al., 2017). In addition, several studies have demonstrated the interaction of CaMKII with actin polymerization (Fink et al., 2003; Kim et al., 2015), as well as with enzymes of the Rho/Rac family controlling spine dynamics, turnover and morphology (Murakoshi et al., 2011).

It is well established that the Bla-IC pathway contributes to the formation and retention of CTA memory (Escobar and Bermúdez-Rattoni, 2000; Rodríguez-Durán et al., 2011, 2017; Rodríguez-Durán and Escobar, 2014). CTA and IC-LTP share similar molecular mechanisms, such as NMDA receptor dependence (Escobar et al., 1998a, 2002; Rodríguez-Durán and Escobar, 2014), activation of extracellular signal-regulated kinase-1/2 (ERK1/2), immediately-early gene expression (Jones et al., 1999), and protein synthesis dependence (Moguel-González et al., 2008; Rodríguez-Durán et al., 2011). However, the identity of the actors involved in CTA-LTM maintenance remains

largely unknown. Our present findings reveal that CaMKII is a relevant actor for the maintenance of CTA-LTM, since intracortical microinfusion of myr-CaMKIINtide 24 h after CTA acquisition lead to memory impairment during LTM aversion test, performed 72 h after CTA acquisition. Importantly, the effect of CaMKII inhibition on CTA-LTM was specific to CS-US association, since the memory deficit was not present when rats were pseudotrained in CTA.

In this line of ideas, it has been shown that training rats in the Morris water maze positively correlates with an increase in hippocampal CaMKII autonomous activity (Tan and Liang, 1996). In addition, learning of a step-down inhibitory avoidance task increases hippocampal CaMKII activity (Cammarota et al., 1998). Recently, it has been found that expression of a dominant-negative form of CaMKII lead to a strong reduction in spatial memory of mice that persisted even after this form was no longer expressed (Rossetti et al., 2017). In the case of IC, it has been shown that exposure to a novel taste lead to an increase in CaMKII autophosphorylation for up to 3 h after taste consumption. In addition, application of a selective CaMKII inhibitor in the IC 25 min after saccharine consumption attenuated CTA memory tested 3 or 5 h after the establishment of the association (Adaikkan and Rosenblum, 2015). On the other hand, studies involving genetic disruption of CaMKII activity have shown that homozygous αCaMKII mutant mice present deficits in spatial learning (Silva et al., 1992), however they can be overcome with extended training (Elgersma et al., 2002). This compensation cannot be found in mice with a threonine-to-alanine point mutation of CaMKII, preventing the autophosphorylation of the kinase (Giese et al., 1998; Need and Giese, 2003). Moreover, CaMKII autophosphorylation-deficient mutant mice exhibit impaired *de novo* gene transcription required for contextual fear memory consolidation (von Hertzen and Giese, 2005). In a pioneer study Frankland and colleagues, demonstrated that while mice heterozygous for a CaMKII null mutation have normal memory retention for contextual fear and water maze tasks 1–3 days after training, these animals were amnesic when tested 10–50 days post-training, suggesting a role for CaMKII not only in consolidation but also in the maintenance of memory (Frankland et al., 2001). Since the association between CaMKII and GluN2B has been proposed as a substrate for memory maintenance, mice with a mutation that prevents the formation of the CaMKII/GluN2B complex show memory impairment in the Morris water maze when tested at 1 or 3 days after the last training session (Halt et al., 2012).

In summary, our present results show that CaMKII inhibition in the IC during the late-phase of *in vivo* LTP of the Bla-IC projection, described as a necessary pathway for acquisition and storage of CTA memory, blocks the potentiation maintenance. In the same manner, inhibition of CaMKII in the IC 24 h after CTA acquisition impairs the CTA memory persistence. Together these results indicate that CaMKII is a central key component for the maintenance of neocortical synaptic plasticity as well as for persistence of CTA-LTM.

AUTHOR CONTRIBUTIONS

YJ-M: Acquisition, analysis, and interpretation of data; drafting the article and revising it critically for important intellectual content. LR-L: Acquisition, analysis, and interpretation of data. ME: Conception and design; analysis and interpretation of data, drafting the article and revising it critically for important intellectual content.

ACKNOWLEDGMENTS

This research was supported by PAPIIT IN215816 and CONACYT 367853 and 474. We thank Esteban Urrieta Chávez for careful reading of the article.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 10 January 2017
doi: 10.3389/fphar.2016.00539

Verapamil Parameter- and Dose-Dependently Impairs Memory Consolidation in Open Field Habituation Task in Rats

*Natalija Popović[1,2], Verónica Giménez de Béjar[2,3], María Caballero-Bleda[1,2] and Miroljub Popović[1,2]**

[1] Department of Human Anatomy and Psychobiology, Faculty of Medicine, University of Murcia, Murcia, Spain, [2] Instituto Murciano de Investigación Biosanitaria, Virgen de la Arrixaca, Murcia, Spain, [3] Department of Neurology, Santa Lucía University General Hospital, Cartagena, Spain

The purpose of the present study was to examine the effects of the phenylalkylamine class of the L-type voltage-dependent calcium channel antagonist, verapamil (1.0, 2.5, 5.0, or 10 mg/kg i.p.), administered immediately after the acquisition task, on memory consolidation of the open field habituation task, in male Wistar rats. On the 48 h retested trial, all tested parameters (ambulation in the side wall and in the central areas, number of rearing, time spent grooming and defecation rate) significantly decreased in the saline treated animals. A significant decrease of rearing was observed in all verapamil treated groups. On the retention day, the ambulation in the side wall and central areas significantly decreased in the animals treated with 1 mg/kg and 10 mg/kg of verapamil, while the time spent grooming and the defecation rate significantly decreased only in the group treated with 1 mg/kg of verapamil. According to the change ratio scores that correct the individual behavioral baseline differences during initial and final sessions, habituation deficit was found in animals treated with verapamil as follows: ambulation along the side wall area (1, 2.5, and 5 mg/kg), number of rearing (all used dose) and time spent grooming (2.5, 5, and 10 mg/kg). In conclusion, the present data suggest that the post-training administration of verapamil, parameter- and dose-dependently, impairs the habituation to a novel environment.

Keywords: habituation, memory consolidation, open field, verapamil, rat

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Thomas Van Groen, University of Alabama at Birmingham (UAB), USA
Enrico Patrono, University of Toyama, Japan

***Correspondence:**
Miroljub Popović
miroljub@um.es

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *07 December 2016*
Accepted: *26 December 2016*
Published: *10 January 2017*

Citation:
Popović N, Giménez de Béjar V, Caballero-Bleda M and Popović M (2017) Verapamil Parameter- and Dose-Dependently Impairs Memory Consolidation in Open Field Habituation Task in Rats. Front. Pharmacol. 7:539. doi: 10.3389/fphar.2016.00539

INTRODUCTION

Three different subfamilies of calcium (Ca_v) channels have, thus far, been defined and named Ca_v1, Ca_v2, and Ca_v3 (Ertel et al., 2000; Catterall et al., 2005; Hofmann et al., 2014). The Ca_v1 subfamily (Ca_vl.l–Ca_vl.4) includes channels which mediate L-type Ca^{2+}currents, whereas the Ca_v2 subfamily ($Ca_v2.1$–$Ca_v2.3$) comprises channels which originate P/Q-, N-, and R-type Ca^{2+}currents, respectively. Lastly, the Ca_v3 subfamily (Cav3.1–Cav3.3) includes channels which mediate T-type Ca^{2+} currents (Ertel et al., 2000; Catterall et al., 2005; Hofmann et al., 2014).

Molecular genetics approaches clearly suggest essential implication of the $Ca_v1.3$ but not the $Ca_v1.2$ subunit of the L-type voltage-gated calcium channels (LVGCCs), in the process of memory consolidation of contextual fear conditioning in mice (McKinney and Murphy, 2006; McKinney et al., 2008). However, the effects of several classes of LVGCC antagonists,

such as dihydropyridines (e.g., nimodipine, nicardipine, and nifedipine), benzothiazepines (e.g., diltiazem), phenylalkylamines (e.g., verapamil), and diphenylalkylamines (e.g., flunarizine), on memory consolidation in young mice and rats, are controversial. It has been found that nimodipine does not affect the memory consolidation of rats tested in the simple learning association (Isaacson et al., 1989), as well as of mice tested in contextual fear conditioning (Suzuki et al., 2004). Calcium channel blockers that bind principally to dihydropyridine receptors (nimodipine, nifedipine, and amlodipine) do facilitate memory consolidation of mice tested in passive avoidance, linear maze, and elevated plus-maze tasks (Quartermain et al., 1993, 2001; Biala et al., 2013). On the other hand, diltiazem and flunarizine improve memory consolidation of mice tested in passive avoidance and linear maze (Quartermain et al., 1993, 2001) but not in the elevated plus-maze task (Biala et al., 2013). Studies in mice demonstrated that systemic post-training treatment with verapamil does not affect memory consolidation in the passive avoidance task (Quartermain et al., 1993; Masoudian et al., 2015), but improves retention in linear maze and elevated plus maze tasks (Biala et al., 2013). Noting the lack of data related to the effect of LVGCC antagonists on memory consolidation in rats, the aim of the present study was to evaluate the effects of verapamil post-training treatment on memory retention, in the open field habituation task.

MATERIALS AND METHODS

Experimental Animals

Experiments were carried out on male Wistar rats, weighing 200–250 g. The animals were housed in standard Makrolon cages on sawdust bedding. They were kept in an air-conditioned room (20 ± 1°C), at 30% humidity and under a 12 h light/12 h dark cycle (lights on from 08:00 to 20:00 h). Food and tap water were available *ad libitum*. One week before the experimental procedure, the rats were handled daily for 5 min each. The behavioral tests were performed during the light period (16:00–20:00 h).

All procedures related to the animal maintenance and experimentation were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the guidelines issued by the Spanish Ministry of Agriculture, Fishing and Feeding (Royal Decree 1201/2005 of October 21, 2005) and were approved by the Animal Ethics Committee of the University of Murcia. Efforts were made to minimize the number of animals used, as well as their suffering.

Drugs

The saline solution of verapamil (Sigma, St. Louis, MO, USA) was administered intraperitoneally at the dose of 1, 2.5, 5, or 10 mg/kg. Control animals were treated with physiological saline at the dose of 1 ml/kg body weight. Eight animals were assigned in each tested group.

Open Field Test

The open field test was performed in a square white plywood box (100 cm × 100 cm × 40 cm). The floor was divided into 25 (20 × 20 cm) squares. On day 1, the rats were initially placed at one of the four corners of the box and their behavior was monitored during 10 min. After that, the rats were removed from the open field, drug administered and returned to their home cage. Forty-eight hours later, the retention test was given. The open field test was performed under 300 lux light intensity and recorded using a video camera to enable subsequent evaluation. The apparatus was cleaned with 70% ethanol before each animal was tested. Eight animals were assigned in each tested group.

In the open field test, the *ambulation along the side wall area* (number of outer squares entered), the *ambulation in the central area* (number of inner squares entered), the *number of rearing* (standing on the hind legs, with or without contact with the sides of the arena), the *time spent frozen* (time that the animal spent immobile), the *time spent in grooming* (time that the animal spend licking, scratching or cleaning any part of its head or body) and the *defecation* (number of fecal boli deposited) were recorded. With the aim to correct the individual baseline differences in the studied parameters, we used a change ratio score to compare behavior during initial and final sessions. This score is calculated according to the Bolivar (2009) as follows: day 2 parameter value/(day 1 parameter value + day 2 parameter value). Thus, the change ratio score will approach 0.5 if no change in behavior has occurred, i.e., no habituation. If the ratio approaches 0, there is evidence of habituation. If the ratio approaches 1.0, the tested behavioral parameter has actually increased from the initial to the final time periods.

Statistical Analysis

The statistical analysis was made using the SPSS 19.0 statistical package. The data were analyzed with the General Linear Model (GLM) repeated measures analysis and presented as mean ± standard error of the mean (SEM). If the GLM showed significant differences between groups, a *post hoc* analysis was performed. The group differences on the acquisition trial were analyzed by the two-tailed Student's *t*-test for independent samples. The two-tailed Student's *t*-test for paired-samples was used for comparison of the data between the acquisition and the retention trial. The habituation scores were analyzed by the one-way ANOVA test, followed by the two-tailed Student's *t*-test for independent samples. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Open Field Test

Only one animal from the saline treated group and four animals from the verapamil treated groups displayed freezing behavior (data not showed). The rest of the data from the open field test are presented in the **Figure 1**.

The GLM repeated measure analysis showed a significant *effect of time* on ambulation in the side wall area ($F_{(1)} = 47.577$,

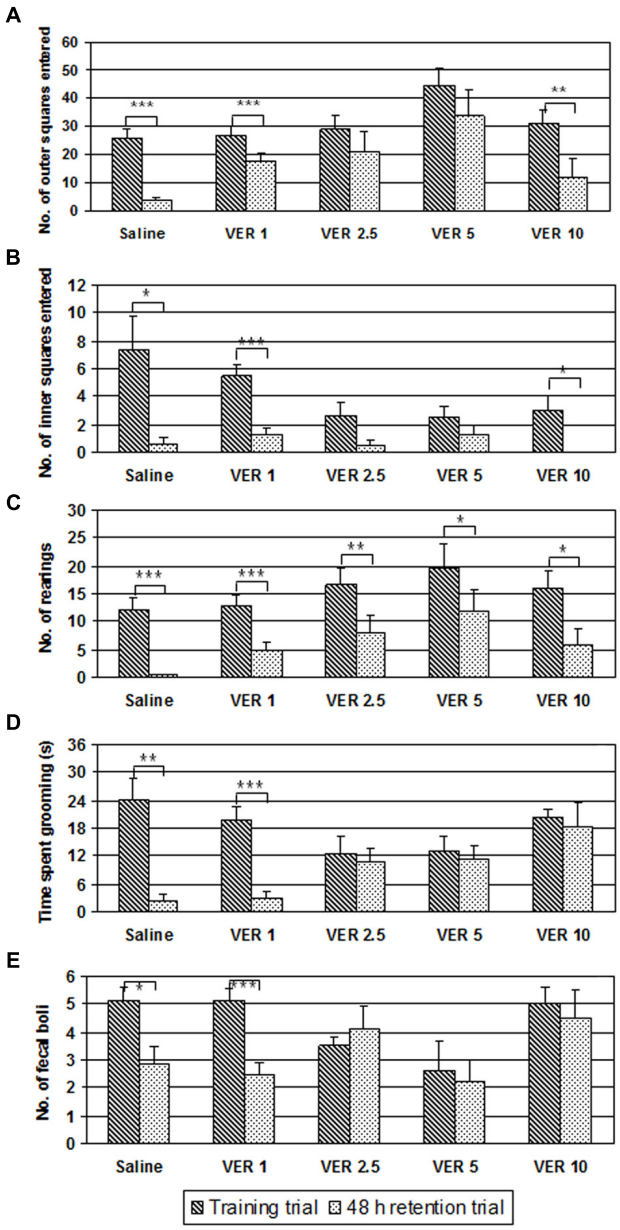

FIGURE 1 | Effect of 1, 2.5, 5, and 10 mg/kg of verapamil (VER-1, VER-2.5, VER-5, and VER-10, respectively), administered intraperitoneally, immediately after the acquisition trial, on ambulation in the side wall area (A), ambulation in the central area **(B)**, number of rearing **(C)**, time spent grooming **(D)**, and defecation **(E)**, in the open field habituation task. The data are presented as mean ± standard error of the mean (SEM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. acquisition trial.

$p < 0.001$), ambulation in the central area ($F_{(1)} = 42.976$, $p < 0.001$), number of rearing ($F_{(1)} = 64.390$, $p < 0.001$), time spent grooming ($F_{(1)} = 19.682$, $p < 0.001$), and defecation ($F_{(1)} = 6.712$, $p < 0.05$). There was significant *effect of group* on ambulation in the side wall area ($F_{(4)} = 3.207$, $p < 0.05$) but not on ambulation in the central area ($F_{(4)} = 1.987$, $p > 0.05$), number of rearing ($F_{(4)} = 2.013$, $p > 0.05$), time spent grooming ($F_{(4)} = 2.006$, $p > 0.05$), and defecation ($F_{(4)} = 2.454$, $p > 0.05$). There was significant *effect of interaction time × group* on ambulation in the central area ($F_{(4)} = 3.261$, $p < 0.05$) and on the time spent grooming ($F_{(2)} = 4.785$, $p < 0.01$) but not on ambulation in the side wall area ($F_{(4)} = 2.128$, $p > 0.05$), number of rearing ($F_{(4)} = 0.372$, $p > 0.05$), and defecation ($F_{(4)} = 2.390$, $p > 0.05$).

In the acquisition trial of the open field test, there were no significant differences between groups in the ambulation in the side wall area ($F_{(4)} = 2.591$, $p > 0.05$), ambulation in the central area ($F_{(4)} = 2.542$, $p > 0.05$), number of rearing ($F_{(4)} = 1.061$, $p > 0.05$), time spent grooming ($F_{(4)} = 2.233$, $p > 0.05$), and defecation rate ($F_{(4)} = 2.604$, $p > 0.05$). The two-tailed Student's t-test for paired-samples showed that ambulation in the side wall (**Figure 1A**) and in the central (**Figure 1B**) areas, significantly decreased on the retention day, in the saline group ($t = 6.677$, $df = 7$, $p < 0.001$; $t = 3.441$, $df = 7$, $p < 0.05$, respectively) and in the animals treated with verapamil at the dose of 1 mg/kg ($t = 5.105$, $df = 7$, $p < 0.001$; $t = 6.298$, $df = 7$, $p < 0.001$, respectively) and 10 mg/kg ($t = 3.486$, $df = 7$, $p < 0.01$; $t = 2.722$, $df = 7$, $p < 0.05$, respectively) but not in the animals treated with verapamil at the dose of 2.5 ($t = 2.004$, $df = 7$, $p > 0.05$; $t = 1.945$, $df = 7$, $p > 0.05$, respectively) and 5 mg/kg ($t = 1.708$, $df = 7$, $p > 0.05$; $t = 2.236$, $df = 7$, $p > 0.05$, respectively). The two-tailed Student's t-test for paired-samples showed that the number of rearing ($t = 5.276$, $df = 7$, $p < 0.001$; $t = 5.420$, $df = 7$, $p < 0.001$; $t = 3.804$, $df = 7$, $p < 0.01$; $t = 2.396$, $df = 7$, $p < 0.05$; $t = 3.155$, $df = 7$, $p < 0.05$) (**Figure 1C**) significantly decreased on the retention day in both the saline- and verapamil-treated (1, 2.5, 5, and 10 mg/kg) animals, respectively. On the retention day, the time spent grooming (**Figure 1D**) and the defecation rate (**Figure 1E**) significantly decreased in the groups treated with saline ($t = 4.376$, $df = 7$, $p < 0.01$; $t = 3.000$, $df = 7$, $p < 0.05$, respectively) and verapamil at the dose of 1 mg/kg ($t = 6.120$, $df = 7$, $p < 0.001$; $t = 8.104$, $df = 7$, $p < 0.001$, respectively) but not in animals treated with verapamil at the doses of 2.5 mg/kg ($t = 0.501$, $df = 7$, $p > 0.05$; $t = -0.804$, $df = 7$, $p > 0.05$, respectively), 5 mg/kg ($t = 0.367$, $df = 7$, $p > 0.05$; $t = 0.351$, $df = 7$, $p > 0.05$, respectively), and 10 mg/kg ($t = 0.346$, $df = 7$, $p > 0.05$; $t = 0.408$, $df = 7$, $p > 0.05$, respectively).

The one-way ANOVA test revealed significant differences in the change ratio score for ambulation in the side wall area ($F_{(4)} = 7.097$, $p < 0.001$), number of rearing ($F_{(4)} = 5.169$, $p < 0.01$) and time spent grooming ($F_{(4)} = 5.616$, $p < 0.001$) but not in ambulation in the central area ($F_{(4)} = 1.266$, $p > 0.05$) and defecation rate ($F_{(4)} = 1.539$, $p > 0.05$). The *post hoc* analysis showed that the change ratio score for ambulation in the side wall area was significantly higher in animals treated with verapamil in doses of 1, 2.5, and 5 mg/kg than in those treated with saline ($t = -10.104$, $df = 14$, $p < 0.001$; $t = -4.042$, $df = 14$, $p < 0.001$; $t = -4.091$, $df = 14$, $p < 0.001$, respectively) or 10 mg/kg of verapamil ($t = 3.242$, $df = 14$, $p < 0.01$; $t = -2.154$, $df = 14$, $p < 0.05$; $t = 2.195$, $df = 14$, $p < 0.05$, respectively) (**Figure 2**). The change ratio score for the number of rearing was significantly higher in the animals treated with verapamil (1, 2.5, 5, and 10 mg/kg), compared to those treated with saline ($t = -6.216$, $df = 14$, $p < 0.001$; $t = -3.501$, $df = 14$, $p < 0.01$; $t = -4.219$,

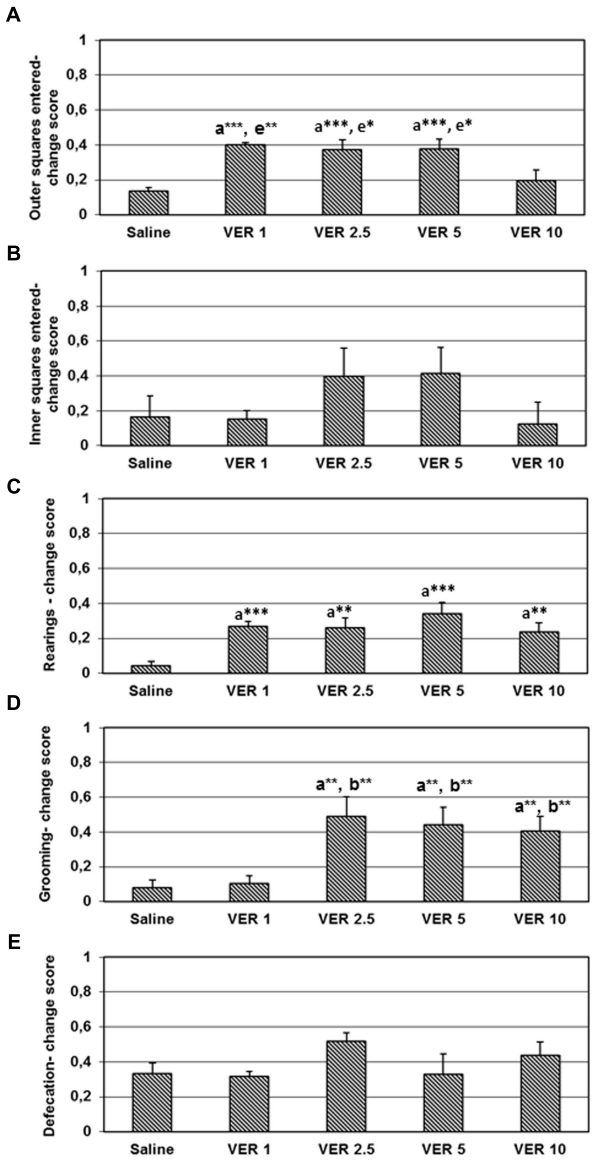

FIGURE 2 | Effect of 1, 2.5, 5, and 10 mg/kg of verapamil (VER-1, VER-2.5, VER-5, and VER-10, respectively), administered i.p. immediately after the acquisition trial, on habituation score in ambulation in the side wall area (A), ambulation in the central area **(B)**, number of rearing **(C)**, time spent grooming **(D)**, and defecation **(E)**, in the open field habituation task. The data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. acquisition trial. ^{a,b,e} vs. saline, VER-1 or VER-10 groups, respectively.

$df = 14$, $p < 0.001$; $t = -3.349$, $df = 14$, $p < 0.01$, respectively) (**Figure 2**). The animals treated with verapamil in doses of 2.5, 5, and 10 mg/kg have significantly higher change score for time spent grooming than the animals treated with saline ($t = -3.307$, $df = 14$, $p < 0.01$; $t = -3.289$, $df = 14$, $p < 0.01$; $t = -3.431$, $df = 14$, $p < 0.01$, respectively) and verapamil in dose of 1 mg/kg ($t = -3.129$, $df = 14$, $p < 0.01$; $t = -3.092$, $df = 14$, $p < 0.01$; $t = -3.206$, $df = 14$, $p < 0.01$, respectively) (**Figure 2**).

DISCUSSION

In the present study, we demonstrated that verapamil, dose- and parameter-depending, affects habituation in the open field test, one of the most elementary forms of non-associative hippocampal-dependent learning (Leussis and Bolivar, 2006). Although, the animals treated with verapamil (in all tested doses), significantly reduced the rearing component of the exploratory behavior on the retention trial, the level of habituation was impaired compared to the saline treated animals. In contrast, the middle-used doses of verapamil (2.5 and 5 mg/kg) did not significantly decrease the ambulation component of the exploratory behavior, indicating, on this parameter, the inverted U-shape dose-response curve of its action. However, the habituation score of ambulation in the side wall area (verapamil 1, 2.5, and 5 mg/kg) was significantly impaired, but not the habituation score of ambulation in the central area. Regarding to grooming and defecation, a significant decrease in those parameters was found only in animals treated with verapamil in the dose of 1 mg/kg. In spite of this, the habituation score of grooming was significantly lower in animals treated with verapamil (2.5, 5, and 10 mg/kg) compared to the saline treated animals. The present data support our previous findings that mechanisms of habituation are complex and that each component could be differently affected by the drugs (Popović, 2014).

It has been demonstrated that verapamil treatment at the doses of 1, 2, and 10 mg/kg, but not at the dose of 20 mg/kg, significantly improves the consolidation of spatial memory in the linear maze task in mice (Quartermain et al., 2001). Similarly, verapamil at the doses of 5 and 10 mg/kg, but not at the doses of 2.5 and 20 mg/kg, displays an enhancement effect on the consolidation of spatial memory in the elevated plus maze task in mice (Biala et al., 2013), resembling the inverted U-shape dose-response curve of its action. It has been suggested that the inverted U-shape dose-response curve of the verapamil action, could be due to modulation rather than to a complete blockade of LVGCCs (Biala et al., 2013).

The mechanism by which verapamil facilitates or impairs memory consolidation in different tasks, is still unclear. Although classified as L-type calcium channel blocker (being $Ca_v1.2$ channels more sensitive than the $Ca_v1.3$ ones), verapamil blocks $Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$, and $Ca_v3.2$ channels too (Ishibashi et al., 1995; Cai et al., 1997; Dobrev et al., 1999; Tarabova et al., 2007; Kuryshev et al., 2014). Given that the systemic administration of verapamil (range dose 1–10 mg/kg) does not affect the open field behavior (Rogério and Takahashi, 1990; Popović, 1997), the effect of verapamil on memory consolidation of the open field habituation, seems to be independent on the anxiety level. In this line, in $Ca_v1.3$ knockout mice the open field behavior was not changed (Busquet et al., 2010), while only severe reduction in the $Ca_V1.2$ activity, in the mouse forebrain, enhanced anxiety-like behaviors in open field (Lee et al., 2012). As far to our knowledge, there are no data analyzing the implication of these subunits in the consolidation of the open field habituation.

In view of the fact that verapamil can block large conductance calcium-activated potassium channels (BK channel)

(Harper et al., 2001) and that habituation of the exploratory locomotor behavior in the open field is not affected in the BK knock-out mice (Typlt et al., 2013), it could be less probable that the effect of verapamil be partially due to the action on these channels. It is possible that verapamil, through its action as an antagonist of P-glycoprotein transporters or multidrug resistance proteins (Pajeva and Wiese, 2002), impairs memory consolidation in open field task, since P-glycoprotein knockout mice display lower level of habituation than the wild-type mice (Schoenfelder et al., 2012).

CONCLUSION

As far to our knowledge, the present data represent the first evidence that post-training verapamil administration produces parameter- and dose-dependent impairment of habituation in the open field task in rats. Moreover, the present findings resembling the inverted U-shape dose-response curve of verapamil action.

ETHICS STATEMENT

All procedures related to the animal maintenance and experimentation were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and the guidelines issued by the Spanish Ministry of Agriculture, Fishing and Feeding (Royal Decree 1201/2005 of October 21, 2005) and were approved by the Animal Ethics Committee of the University of Murcia. Efforts were made to minimize the number of animals used, as well as their suffering.

AUTHOR CONTRIBUTIONS

All authors (NP, VGdB, MC-B, and MP) contributed to the design of the study, wrote the protocol and managed the literature searches. Authors NP, VGdB, and MP performed the experiments and undertook the statistical analysis. All authors (NP, VGdB, MC-B, and MP) contributed to drafting the work and have approved the final manuscript.

FUNDING

Funding for this study was provided by the Health Council of Murcia Region, Spain (MC) and the Spanish Ministry of Economy and Competitiveness (BFU2014-57516-P; LPL, JLF). The funding source had no further role in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 09 February 2017
doi: 10.3389/fphar.2017.00064

N-Butylphthalide Improves Cognitive Function in Rats after Carbon Monoxide Poisoning

Ming-Jun Bi[1,2], Xian-Ni Sun[2], Yong Zou[1], Xiao-Yu Ding[1,3], Bin Liu[4], Yue-Heng Zhang[5], Da-Dong Guo[6] and Qin Li[1]**

[1] Department of Integration of Chinese and Western Medicine, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China, [2] Emergency Centre, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, China, [3] Department of Integration of Chinese and Western Clinical Medicine, Qingdao University Medical College, Qingdao, China, [4] The Second Clinical Medical College, Shandong University of Traditional Chinese Medicine, Jinan, China, [5] Department of Clinical Medicine, Binzhou Medical University, Yantai, China, [6] Eye Institute, Shandong University of Traditional Chinese Medicine, Jinan, China

Cognitive impairment is the most common neurologic sequelae after carbon monoxide (CO) poisoning, and the previous investigations have demonstrated that *N*-Butylphthalide (NBP) could exert a broad spectrum of neuroprotective properties. The current study aimed to investigate the effect of NBP on cognitive dysfunction in rats after acute severe CO poisoning. Rats were randomly divided into a normal control group, a CO poisoning group and a CO+NBP group. The animal model of CO poisoning was established by exposure to CO in a chamber, and then all rats received hyperbaric oxygen therapy once daily, while rats in CO+NBP group were administered orally NBP (6 mg/ 100g) by gavage twice a day additionally. The results indicated that CO poisoning could induce cognitive impairment. The ultrastructure of hippocampus was seriously damaged under transmission electron microscopy, and the expressions of calpain 1 and CaMK II proteins were significantly elevated after CO exposure according to the analysis of immunofluorescence staining and western blot. NBP treatment could evidently improve cognitive function, and maintain ultrastructure integrity of hippocampus. The expression levels of both calpain 1 and CaMK II proteins in CO+NBP group were considerably lower than that of CO poisoning group ($P < 0.05$). Taken together, this study highlights the molecular mechanism of cognitive dysfunction in rats after CO exposure via the upregulation of both calpain 1 and CaMK II proteins. The administration of NBP could balance the expressions of calpain 1 and CaMK II proteins and improve cognitive function through maintaining ultrastructural integrity of hippocampus, and thus may play a neuroprotective role in brain tissue in rats with CO poisoning.

Keywords: Ca^{2+}/calmodulin dependent protein kinase II, Calpain 1, CO poisoning, cognitive function, *N*-butylphthalide, rat

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Fathi M. Sherif, University of Tripoli, Libya
Yunliang Guo, Qingdao University Medical College, China
Leonardo Cocito, University of Genoa, Italy

****Correspondence:***
Da-Dong Guo
dadonggene@163.com
Qin Li
liqin701015@163.com

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *20 October 2016*
Accepted: *30 January 2017*
Published: *09 February 2017*

Citation:
Bi M-J, Sun X-N, Zou Y, Ding X-Y, Liu B, Zhang Y-H, Guo D-D and Li Q (2017) N-Butylphthalide Improves Cognitive Function in Rats after Carbon Monoxide Poisoning. Front. Pharmacol. 8:64. doi: 10.3389/fphar.2017.00064

INTRODUCTION

From a public health perspective, unintentional carbon monoxide (CO) poisoning is a leading factor of accidental poisoning in the United States, and may be the cause of more than 50% fatal poisonings in many industrial countries (Omaye, 2002; Geraldo et al., 2014). The clinical signs and symptoms associated with CO toxicity effects depend on the concentration and

duration of exposure, ranging from slight headache, nausea, vomiting, shortness of breath, malaise and palpitation, to confusion, unconsciousness, coma and even death (Wright, 2002). There are permanent neurologic problems in 46% of survivor (Choi, 2002; Yogaratnam et al., 2011), and the delayed neurological manifestations, such as cognitive and personality changes, incontinence, psychosis, and parkinsonism, are the most common neurologic sequelae, and develop between 2 days and 8 months later in 10% to 30% of survivors. Many studies have provided magnitude estimates of the lesions affecting cortex, basal ganglia, globus pallidus and white matter changes within the corpus callosum and periventricular region (Chang et al., 2010; Lakhani and Bleach, 2010; Ruth-Sahd et al., 2011). Nevertheless, few investigations evaluate the relationship between the cognitive impairment and hippocampus damage after exposure to CO (Chen et al., 2013). *N*-butylphthalide (NBP), originally extracted from the seeds of Apium graveolens Linn, has displayed a broad spectrum of neuroprotective properties. It has been demonstrated that NBP could efficiently improve cognitive deficits induced by chronic intermittent hypoxia (IH)-hypercapnia exposure (Min et al., 2014), protect cells against ischemic damage via multiple mechanisms including mitochondria associated caspase-dependent and -independent apoptotic pathways both *in vitro* and *in vivo* (Li et al., 2010; Wang et al., 2014), maintain mitochondrial function and balance the expressions of anti-apoptosis genes and pro-apoptosis genes. Meanwhile, the experimental investigations have revealed that NBP administration at the dosage of (15–160 mg/ kg) was safe and reliable via oral or intraperitoneal injection (Xiong et al., 2012; Diao et al., 2014). Moreover, NBP, to some extent, could also participate in the activation of Keap1-Nrf-2/antioxidant response element (ARE) signaling pathway, and thus play neuroprotective roles against brain damage after acute CO poisoning (Li Q. et al., 2015). In the present study, we aimed to investigate the underlying mechanisms of cognitive dysfunction in rat models after exposure to CO, and evaluate the feasibility of NBP treatment on the structural and functional impairment of hippocampus induced by acute severe CO poisoning.

MATERIALS AND METHODS

Ethics Statement

Total of 120 adult healthy male Sprague-Dawley rats (7∼8 weeks, weighing (230 ± 20) g were supplied by Qingdao Academy of Medical Sciences, China. All animal experiments were carried out in strict accordance with the regulations for the Care and Use of Laboratory Animals of the National Institute of Animal Health and the Guidance by the ethics committee of Qingdao University (animal welfare assurance number: 14-0027, Bi et al., 2016), and all possible efforts were made to minimize the pain and discomfort of each animal in accordance with the Animal Care and Use Program Guidelines of China.

Subjects and Groups

In the present study, 120 rats were randomly assigned to three groups: a normal control group (NC group, $n = 40$), a CO poisoning group (CO group, $n = 40$) and an NBP treatment group (CO+NBP group, $n = 40$). Prior to experiments, all rats were housed in a temperature-controlled environment with a 12-h light/dark cycle for 7 days, and had free access to food and water throughout the experiment. Rats in CO group and CO+NBP group suffered from CO exposure to establish an animal model in the animal chamber as described previously (Li et al., 2016), while those in normal control group were permitted to breathe fresh air simultaneously. The subjects with coma, high HbCO concentration (≥40%) during CO inhaling and then conscious restoration after a breath of fresh air were considered as the successful models of acute severe CO poisoning. As a result, the conscious recovery time was (28.6 ± 8.8) min in CO group, and (28.5 ± 8.6) min in CO+NBP group, and there was no significant difference between the two groups ($P > 0.05$). During the whole experiment, rat core temperature was maintained at 36 ∼ 37°C using a heated blanket. Three cases were excluded in the final experimental statistics because of continued coma (one rat) or low HbCO concentration (two rats); meanwhile, another three rats of successful models were admitted in the experiment to perform the following tests.

Treatment and Intervention

N-butylphthalide (chemical formula: $C_{12}H_{14}O_2$, molecular weight: 190.24, purity: 100%) was granted by Shijiazhuang Pharmaceutical Co., Ltd., China. All rats received hyperbaric oxygen therapy within 10 min after conscious restoration (Liu W.C. et al., 2016). Rats in CO+NBP group were administrated 6 mg/100 g NBP by gavage using a stomach tube at 2 h after CO exposure additionally, twice a day for 1 day to 1 month till sacrificed (Li J. et al., 2015; Li Q. et al., 2015), and those in CO group and NC group were given the same dosage of pure olive oil as placebo at the same time.

Evaluation of Neurological Behavior

Morris Water Maze Task

Morris water maze task (Shanghai soft Information Technology Co., Ltd., Model number: XR-XM101) was designed to study spatial learning and memory in all rats enrolled in the present experiment using the method described previously (Ueno et al., 2009). A platform was submerged into a tub (diameter = 130 cm; height = 50 cm; depth = 30 cm) of opaque water. The walls in the room around the water maze were covered with black cloth to create a covered area of 4-by-6 m. Two distal cues were fixed on the black walls. The animals were placed into the water from different locations at the beginning of each trial and performed four trials per session twice a day for 4 days before neurological behavior test. EthoVision XT 9 Software Analysis System was used to record the swimming route and the escape latency to finding the platform in detail. Three rats were removed from the experimental statistics because of their average escape latency far away from others, and another three rats met the experimental requirements were supplemented to the appropriate group. The average escape latency and the number of crossing platform were calculated on days 1, 3, 7, at 2 weeks and 1 month after exposure to CO. Data were expressed as the average values of four trials for each rat in different groups.

Shuttle Box Experimental Score

The change of learning and memory ability in the experimental rats was recorded by the active avoidance response (AAR) index established in the condition. Infrared ray will be emitted from the left and right sides of a shuttle box (model number:10080116012), respectively. If the animal locates in the center of the box at the beginning of the experiment, the shuttle test will end when it shades any light beam from either the left or right sides. If the animal stands in the left box, the test will not terminate until it shades the light beam from the right, and vice versa. Animal finishes the shuttle during the buzzer known as the AAR, while it is called passive avoidance response (PAR) in the electrical stimulation stage. Rats were first handled for 5 min per day to acclimate in the behavioral test room for 1 week prior to the start of behavior testing. In this study, the capacity of learning and memory was expressed as the ratio of AAR, that is, the rate of the completed times of ARR to the total times of test (50 times).

Pathological Changes in Hippocampus

Preparation of Paraffin Sections and Hematoxylin-Eosin (HE) Staining

Four rats in each subsection were deeply anesthetized by intraperitoneal injection of 3% pentobarbital, and were perfused with 0.9% sodium chloride and 4% formaldehyde solution 200 ml transcardially at different time points mentioned above. Immediately upon harvest, brain tissues were taken out from skull, post-fixed in 4% formaldehyde for 2 h, immersed in double distilled water for 4 h, and dehydrated in gradient ethanol, transparented in dimethyl benzene, finally embedded in paraffin. Coronal sections were cut at 7 μm thicknesses through the hippocampus consecutively with a microtome (LEICA-RM 2015, Shanghai Leica Instruments Corporation, China) and adhered on the slides prepared with poly-L-lysine, then stored at 4°C. Paraffin sections were stained with HE solution as general procedure and pathological changes of the different areas (including CA1 and CA3) in hippocampus were observed under a light microscope.

Transmission Electron Microscopy (TEM)

To observe the ultrastructural changes of hippocampus in rats after exposure to CO, TEM was applied in the present study. Four animals in each group were deeply anesthetized and the hippocampus tissues were separated from the whole brain carefully and rapidly in a matrix surrounded by cold ice and cut into 1 mm × 1 mm × 1 mm pieces, then immersed in a fixative solution (2.5% glutaraldehyde in 0.1 mmol/l sodium cacodylate, pH 7.4) for 3 h at room temperature. After post-fixation in 1% Osmium tetroxide (pH 7.4) for 2 h at 4°C, the hippocampal pieces were embedded in epoxy resin Epon 812 and cut into ultrathin sections of 50 nm using an ultramicrotome (Leica EM UC6, Germany) on polyvinyl formal at 4°C for preservation. The slices were then immersed in the saturated alcohol solution containing 3% acetic acid uranium (pH = 3.5) in a clean culture dish and dyed for 30 min, followed by 6% lead citrate solution for the ultrastructure observation under a TEM (JEM-1200EX, Japan).

Golgi Staining

Four rats in each group were deeply anesthetized and perfused transcardially with sodium chloride and formaldehyde solution at the indicated time as described above, and then the whole brain were taken out, immersed thoroughly in Golgi mordant dyeing for 7 days at room temperature, followed by immersion in 30% sucrose solution for 48 h at 4°C in a dark environment. The brain tissue was cut into slices at 100 μm thickness and mounted on the anti-off load glass section for Golgi staining. Sections were rinsed thoroughly with triple-distilled water and aqueous ammonia solution (ammonia: distilled water 3:1) for 10 min and then in 1% sodium thiosulfate solution prepared freshly for another 10 min away from light at 26°C. Under a 1000-fold-light microscope, the number of dendritic spines of neurons in CA1 was calculated and analyzed in three brain slices of each rat. The amount of dendritic spines per 10 μm acted on behalf of the dendritic spine density.

Immunofluorescence Staining

The paraffin sections were used to observe the expressions of calpain 1 and CaMK II positive cells by immunofluorescence staining assay, too. The monoclonal antibodies of the two target proteins were granted by Santa Cruz Company. The sections were blocked with sealing buffer (5% normal goat serum and 0.1% Triton X-100 in PBS) for 1 h and incubated with primary antibodies for 2 h at 37°C, (anti-calpain 1, dilution 1: 400; anti- CaMK II, dilution 1: 150), followed by fluorescence secondary antibody. All slides were observed under a fluorescence microscope (Leica, Heidelberger, Germany) in four non-overlapping fields randomly in hippocampus. The optical density (OD) value of positive cells was analyzed using Leica Qwin image processing and analysis system.

Western Blot Analysis

Four rats in each group were deeply anesthetized as described above and then the hippocampal samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking in Tris-buffered saline and Tween 20 solution (TBST) containing 10% skimmed milk powder for 1 h, the PVDF membranes were incubated with primary antibodies (calpain 1 dilution 1: 550; CaMK II dilution 1: 500)for 50 min and horseradish peroxidase (HRP)-conjugated secondary antibody overnight at 4°C. Finally, the membranes were washed fully with PBS and developed in X optical film according to the manufacturer's instructions. The absorbance (A) value of target proteins was analyzed by Bio-Rad 2000 gel imaging system and Quantity one software. The expression level of β-actin in the same sample, as an internal reference, was also detected to normalize the relative A values of target proteins.

Statistical Analysis

Data were presented as mean ± SEM and analyzed using the Graph Prism Program, Version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Differences in the parameters were evaluated using one-way analysis of variance (ANOVA) and least significant

difference (LSD) *t*-test. Values less than 0.05 were considered statistically significant.

RESULTS

Neurobehavioral Changes of Rats in Each Group

In the experiments of positioning navigation and space exploration, we observed that the average escape latency was significantly prolonged in both CO group and CO+NBP group in comparison to that of NC group ($P < 0.05$, **Table 1**). Meanwhile, we also noted that the number of crossing platform in both CO group and CO+NBP group was obviously decreased, and there was a statistical difference compared with that in NC group ($P < 0.05$). The average escape latency in CO+NBP group was shorter than that of CO group, and the number of crossing platform was slightly increased. The difference between the two groups was extremely significant at a late stage of CO poisoning (>1 weeks, $P < 0.05$), yet no significant difference was found at an early stage of poisoning (<3 days, $P > 0.05$). These results suggested that acute CO poisoning could decrease the ability of spatial learning and memory in rats, which may be closely related to the duration of CO exposure. CO+NBP could obviously improve the learning and memory function of rats, and the neuroprotective effect might last for at least 1 month after CO poisoning.

The AAR of rats in CO group were notably decreased compared with that of NC group, and there were significant differences from 1 day to 1 month after CO poisoning ($P < 0.05$). However, the AAR were significantly increased after the administration of NBP, especially at a late stage of CO poisoning (>7 days), and there was statistical significance from 7 days to 1 month after CO poisoning compared to that of CO group ($P < 0.05$, **Figure 1**).

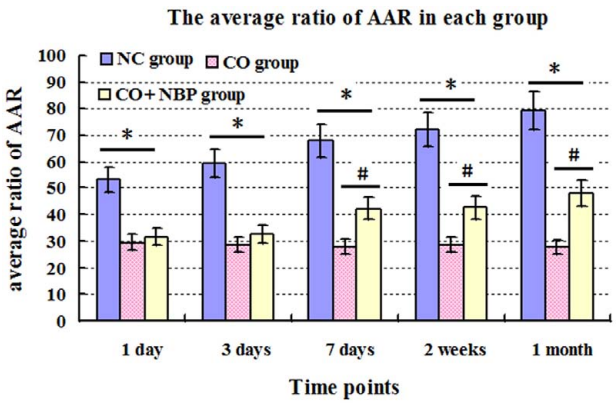

FIGURE 1 | The average ratio of active avoidance response (AAR) in NC, CO poisoning and CO+NBP treatment groups. The average ratio of AAR in rats in CO group was notably decreased compared with those in NC group ($n = 4$, $^{*}P < 0.05$). After administration of NBP, the average ratio of AAR was significantly increased, especially at a late stage of CO poisoning (>7 days), and accompanied by a statistical significance from 7 days to 1 month after CO poisoning compared to that of CO group ($n = 4$, $^{\#}P < 0.05$). $F = 11.528 \sim 20.176$.

Pathological Changes in Hippocampus in Rats

Using HE staining, we found that neurons in CA1 region of hippocampal tissue in NC rats were small, with round or oval shape, and aligned neatly and tightly, while those in CA3 were larger than that in CA1, with clear outline and lightly stained nucleus, and not arranged in order. However, neurons in both CA1 and CA3 regions in CO group were irregular shape, part of which accompanied by pyknosis, even evident shrinkage with spindle-shaped morphology (**Figure 2**), suggesting that CO poisoning can obviously damage the structure of hippocampus. NBP treatment could significantly alleviate the damage of hippocampus in rats after intoxication, and neuronal bodies returned roughly normal dimension and few nuclear karyopyknosis and fragmentation were detected in NBP-treated rats.

Using transmission electron microscopy (TEM), we observed that the exterior contour of hippocampal neurons in NC rats was clear, with big and round nucleus and uniform chromatin. The double nucleus membrane was clear and complete. Mitochondria, rough endoplasmic reticulum, ribosomes, Golgi bodies, and other organelles were rich, and scattered in cytoplasm with structural integrity. In contrast, hippocampal neurons were swelled, nucleus chromatin were condensed and marginalized, mitochondria appeared vacuolization, cristae and membrane were broken, rough endoplasmic reticulum dilated and the ribosomes denuded, and partial cell organelle dissolved or disappeared after CO poisoning (**Figure 3**). The damage degree of hippocampal ultrastructure in CO+NBP group was rather

TABLE 1 | Differences in the average escape latency and the number of crossing platform in Morris water maze performance in different groups.

		n	1 day	3 days	7 days	2 weeks	1 month
NC group	Escape latency (s)	4	44.51 ± 3.51	42.33 ± 3.26	38.42 ± 3.09	36.10 ± 2.67	26.23 ± 2.14
	Number of crossing	4	6.58 ± 0.47	6.68 ± 0.47	7.16 ± 0.51	7.32 ± 0.50	7.42 ± 0.52
CO group	Escape latency (s)	4	55.63 ± 4.28*	53.16 ± 4.25*	49.11 ± 3.90*	45.53 ± 3.52*	39.47 ± 3.11*
	Number of crossing	4	1.32 ± 0.11*	1.32 ± 0.08*	1.32 ± 0.09*	0.98 ± 0.10*	0.98 ± 0.09*
CO+NBP group	Escape latency (s)	4	55.32 ± 4.13*	52.6 ± 4.07*	40.3 ± 3.37*#	38.8 ± 3.12*#	30.1 ± 2.96*#
	Number of crossing	4	1.34 ± 0.13*	1.33 ± 0.10*	1.56 ± 0.11*	2.83 ± 0.18*#	3.21 ± 0.21*#

Compared with NC group, $P < 0.05$; #compared with CO group at the same time point, $P < 0.05$, $F = 10.317 \sim 17.836$.

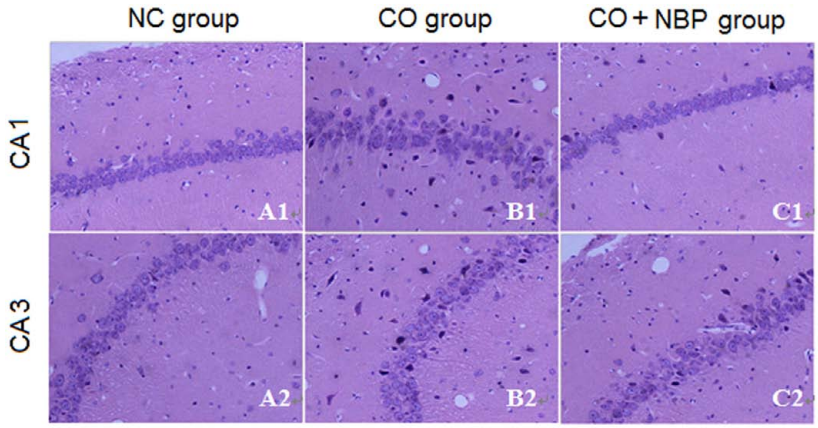

FIGURE 2 | Pathological changes of hippocampus in NC, CO poisoning and CO+NBP treatment groups using HE staining. Neurons in CA1 region were smaller accompanied by either round or oval shape, and were aligned neatly and tightly **(A1)**, while those in CA3 **(A2)** were larger than in CA1 region and did not arrange in order. Meanwhile, few nuclear karyopyknosis and fragmentation were detected in NC samples. Neurons in both CA1 **(B1)** and CA3 **(B2)** regions in CO group were irregular shape, and part of which was pyknosis and shrinking with spindle shape. Neuronal body was roughly normal in both CA1 **(C1)** and CA3 **(C2)** regions in CO+NBP group.

slighter than that of CO group. Double-deckered nuclear membrane was clear, synaptic structure was relatively complete and mitochondria were normal or only slightly swollen with few vacuoles, suggesting that NBP treatment can efficiently improve the ultrastructural damage of hippocampus induced by CO poisoning.

Our study showed that the number of dendritic spines in hippocampal neurons in CO group was lower than that of NC group, but no statistical difference was detected at an early stage of CO poisoning compared with that of NC group (<1 week, $P > 0.05$), whereas a significant difference existed at a late stage of CO poisoning (>2 weeks, $P < 0.05$). Moreover, the number of dendritic spines in CO+NBP group was higher than that in CO group, and it existed statistical difference at a late stage of CO exposure (>2 weeks, $P < 0.05$, **Figure 4**).

The Expressions of Calpain 1 and CaMK II Proteins in Rats after CO Poisoning

Under a fluorescent microscope, a small number of calpain 1 positive cells with different sizes and red fluorescent light were observed in hippocampus in NC subjects. The positive cells were mainly located in cytoplasm, and the amount of calpain 1 positive cells was gradually increased and maintained at relatively higher levels in CO poisoning rats between 3 days and 2 weeks compared to those in NC group ($P < 0.05$). NBP treatment could notably decrease the expressions of calpain 1-positive cells, and the OD value was accordingly dropped compared with that in CO group between 3 days and 2 weeks ($P < 0.05$, **Figure 5**). Furthermore, the same results were also confirmed using western blot assay (**Figure 6**).

Under normal physiological conditions, a basal expression of CaMK II with weak green light in positive cells in hippocampus was observed in NC rats. After exposure to CO, the level of CaMK II increased sharply in a short time, peaked between 1 and 3 days, and then decreased. Nevertheless, it was still higher than that of NC subjects even up to 1 month. Using western blot assay, we found a similar tendency in the change of CaMK II level analyzed by immunofluorescence staining. These results revealed that the overexpression of CaMK II protein might be closely related to hippocampus damage induced by CO poisoning. In addition, we also noted that the number of CaMK II positive cells in CO+NBP treatment group was obviously lower than that in CO group at the same time (**Figure 7**, $P < 0.05$).

The Relationship between Calpain 1 and CaMK II

In order to investigate the relationship between calpain 1 and CaMK II, double immunofluorescence labeling was used in the present study. We found that although the two proteins were mainly in the cell bodies in hippocampus, some calpain 1-positive cells did not show CaMK II immunoreaction. Similarly, not all CaMK II-positive cells exerted calpain 1 immunogenicity in the same view (**Figure 8**). These results suggest that the two proteins can not only co-exist in the same cells, but also express alone in different cells. Further, we found that the expression of calpain 1 was significantly increased after CO poisoning and peaked on 3 days; while the amount of CaMK II positive cells peaked on 1 day, showing a steep rise in a short time period after exposure to CO. Subsequently, the expression levels of these two proteins were gradually decreased. The similar evidence was also assessed and validated by western blotting test, and the result of linear regression analysis clarified the positive quantitative relationship between calpain 1 and CaMK II proteins ($R^2 = 0.8521$), indicating the two proteins would be activated successively after CO poisoning, and then influence the cognitive function of rats.

DISCUSSION

Carbon monoxide poisoning is the leading cause of death by poisoning in industrialized countries, and often leads to

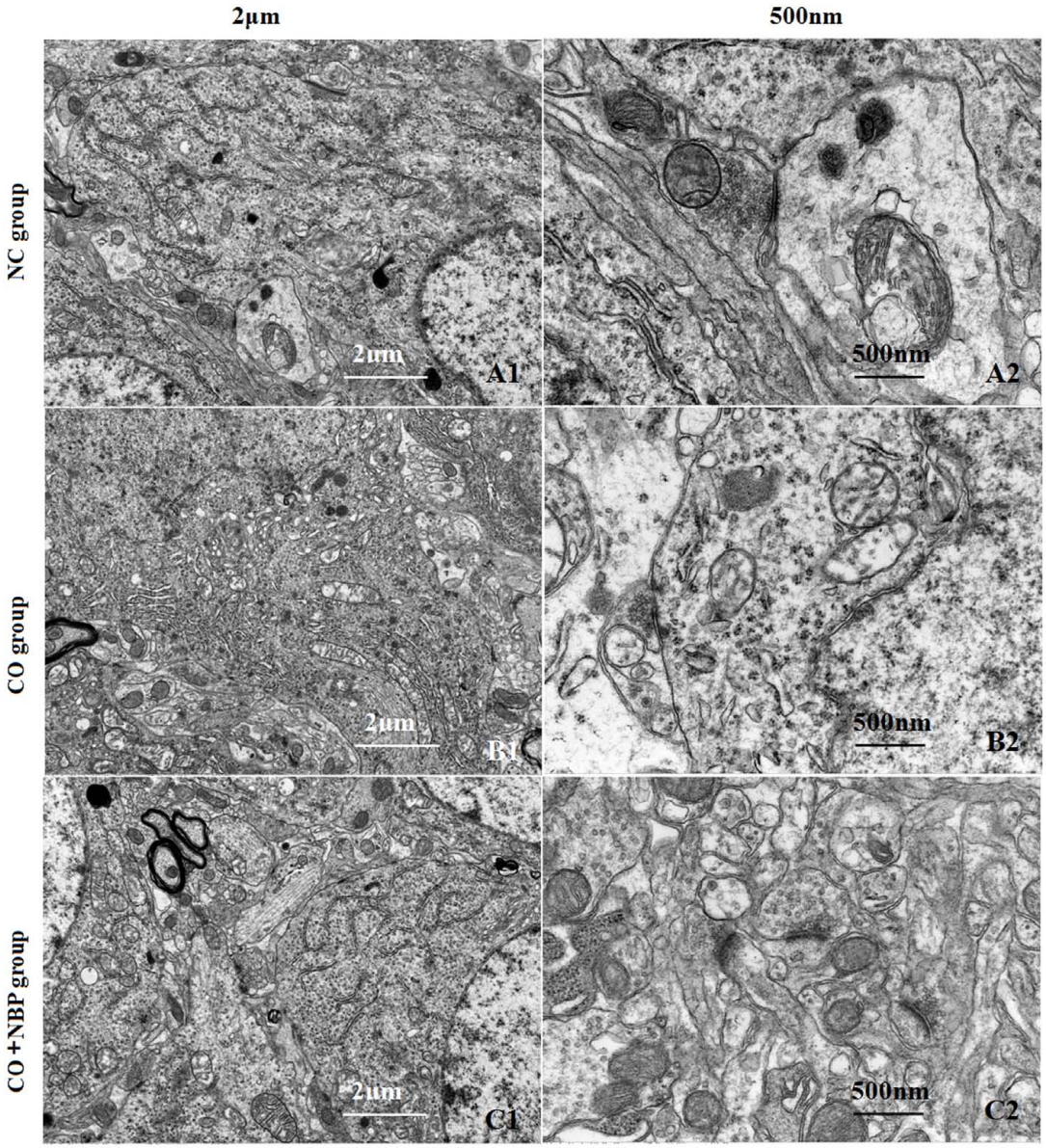

FIGURE 3 | Transmission electron microscopy (TEM) photographs of pathological changes in hippocampus in different groups. The exterior contour of neurons in NC group was clear, with big and round nucleus and uniform chromatin on days 3 **(A1)**. The double nucleus membrane was clear and complete. Organelles were rich and scattered in cytoplasm with structural integrity **(A2)**. Hippocampal neurons were swelled, chromatin in the nucleus was condensed and marginalized, and cristae and membrane were broken. Meanwhile, mitochondria appeared to be vacuolization, and partial cell organelle dissoluted and disappeared in CO poisoning group on days 3 **(B1,B2)**. By contrast, the double-deckered nuclear membrane of neuron was clear, and mitochondria and other organelles were normal or only slightly damaged in CO+NBP group on days 3 **(C1,C2)**.

diffuse hypoxic-ischemic encephalopathy and focal cortical injury, especially in severe acute cases. Cognitive dysfunction is the most common neurological symptoms. In recent decades, many scholars focused on the mechanism, including hypoxia, lipid peroxidation, apoptosis, binding to intracellular proteins and disrupting cellular metabolism, excitotoxicity and cerebral edema, but it is still poorly elucidated about acute brain injury following CO poisoning (Han et al., 2007).

The learning and memory behavior of animal is divided into two aspects. One is based on the memory formation of fear, named passive avoidance and active avoidance, which belongs to simple memory and depends on non-condition reflex and condition reflex. The other is based on the memory formation of visual, known as spatial reference memory, which belongs to advanced memory. Shuttle box test is an important approach for quantitative determination of animal behavior changes in many neurological researches (Lalanza et al.,

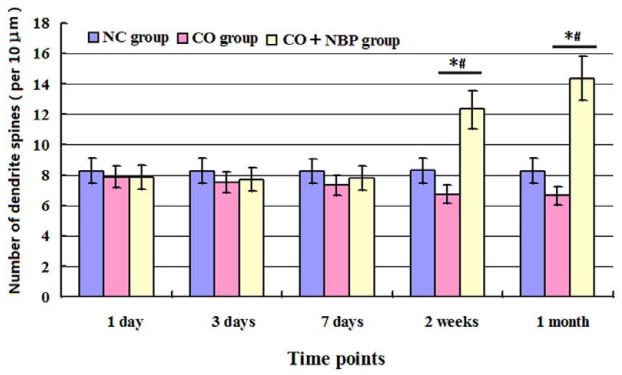

FIGURE 4 | Histogram of the number of dendrite spines per 10 μm in each group using Golgi staining. The number of dendritic spines in hippocampal neurons in CO group was lower than that of NC group, but no statistical difference was detected at an early stage of CO poisoning compared with that of NC group (<1 week, $P > 0.05$), whereas a significant difference existed at a late stage of CO poisoning (>2 weeks, $^{*}P < 0.05$). The administration of NBP could increase the number of dendritic spines, and it existed statistical difference as compared with CO group at a late stage of CO exposure (>2 weeks, $^{\#}P < 0.05$). $F = 13.327 \sim 19.460$.

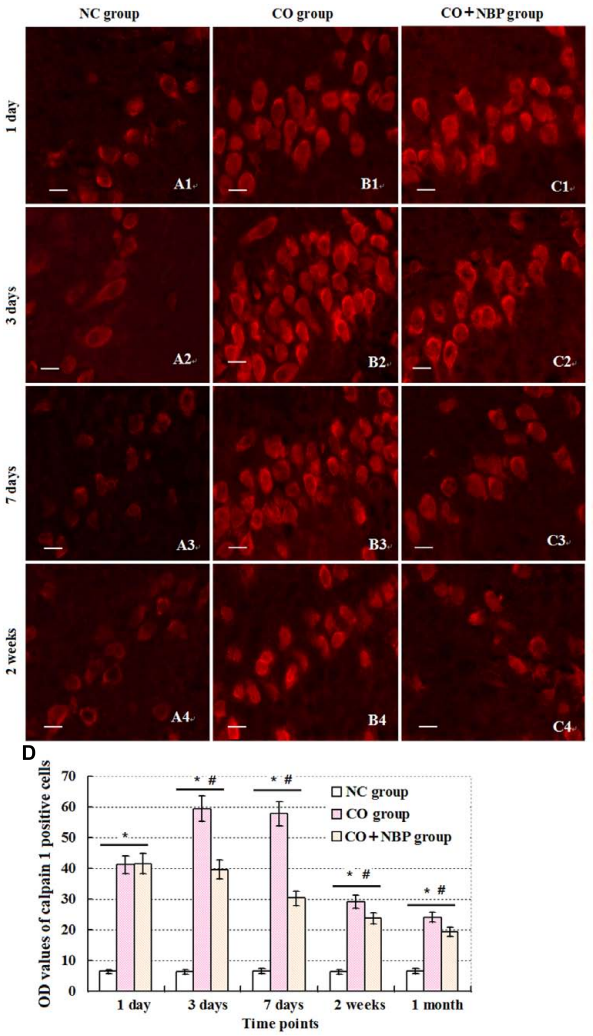

FIGURE 5 | Changes of calpain 1 in hippocampus in each group. Calpain 1 positive cells were observed in hippocampal tissue in NC group, and mainly located in cytoplasm **(A1–C4)**. After exposure to CO, the amount of calpain 1 positive cells was gradually increased, peaked between days 3 and 7, and maintained at relatively higher levels till 2 weeks in contrast to those in NC group ($n = 4$, $P < 0.05$). In contrast, administration of NBP could significantly decrease the expression levels of calpain 1 protein in comparison to that of CO poisoning group at the same time ($n = 4$, $P < 0.05$). **(D)** Histogram of the OD values of calpain 1 positive cells in each group at different time points.*Compared with NC group ($n = 4$, $P < 0.05$); $^{\#}$compared with CO group ($n = 4$, $P < 0.05$). $F = 11.525 \sim 21.618$. Scale bar is 30 μm.

2015; Río-Àlamos et al., 2015), and belongs to the classical conditioned reflex associated with learning, while Morris water maze test mainly for the advanced intelligent activities. Together with shuttle box test and water maze test, we found that after CO poisoning, the active escape latency and the route of escape latency of rats were significantly prolonged, and the number of crossing platform was obviously decreased in rats, suggesting that CO poisoning can damage not only the advanced intelligence activities, but also the classical conditioning reflex.

Hippocampus is the main carrier of learning and memory. Animal experiments have shown that the damage of hippocampus can directly lead to learning and memory disorders. The mechanism of learning and memory is related to the electrophysiological activities of both the long-term potentiation (LTP) and the long-term depression, and the perforant pathway and other loops of hippocampus may be the anatomical basis of LTP in hippocampus (Woodard et al., 2012; Schinazi et al., 2013). Hippocampus, a component of limbic system, is also involved in the pain and emotional responses and other activities (Kim et al., 2012; Martuscello et al., 2012). There are extensive fiber connections between external and internal fibers, which are the structural basis for the complex functional implementation of hippocampus, and the structural abnormalities and pathological changes can lead to some neurological and psychiatric diseases. Golgi staining has been recognized as the most traditional and efficient method of neurological research. Using heavy metal salt staining, neurons and their small dendritic spines were significantly detected, and it is very easy to distinguish the development and death of neurons, the delivery of neurotransmitters and other aspects. In recent years, Golgi staining was used to observe the total length of dendrites and the density of dendritic spines in hippocampal neurons (Martínez-Cerdeño and Noctor, 2014; Peterson et al., 2015). The dendritic spine is closely related to neural plasticity, and the number of dendritic spines directly influences the delivery of neurotransmitters and the ability of learning and memory. In the present study, the results showed that the neuronal ultrastructure was obviously damaged, and the number of dendritic spines in hippocampal neurons was decreased in CO poisoning rats. NBP treatment could efficiently protect the structure integrity of hippocampus, benefit the development of dendritic spines, and elevate the cognitive ability of rats against

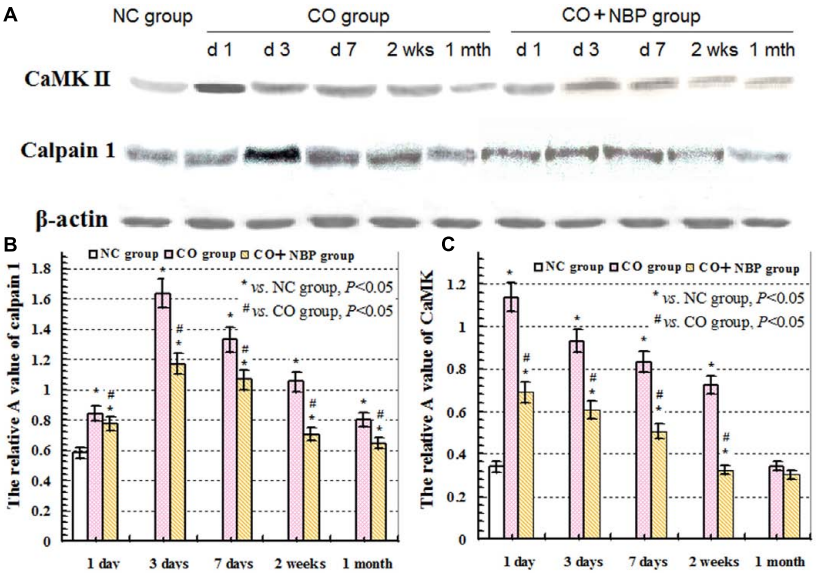

FIGURE 6 | Expressions of CaMK II, calpain 1 and β-actin proteins using western blot assay. (A) The expression changes of CaMK II, calpain 1 and β-actin proteins in each group; **(B)** the relative A value of calpain 1 protein in different group at given time points; **(C)**: the relative A value of CaMK II protein in each group in different time points. * *VS*. NS group, $P < 0.05$; # *VS*. CO group, $P < 0.05$, $F = 12.436 \sim 30.150$.

CO poisoning, and the effect is more evident at a late stage of CO poisoning (>2 weeks). These results suggest that the long-term administration of NBP may be more beneficial to the recovery of learning and memory function in rats after CO poisoning.

The physiological function of hippocampus depends not only on the integrity of structure, but also on the normal quality and quantity of a variety of neurotransmitters. The calcium channels in hippocampal neurons participate in many important physiological functions of nervous system, including LTP and inhibition, learning and memory, etc. The elevated level of calcium ions in nucleus will activate the memory-related genes associated with long-term changes in postsynaptic structure, and which is the mechanism of learning and memory formation (Gurkoff et al., 2013; Nimmrich and Eckert, 2013).

Calpain 1 is a kind of calcium-activated intracellular protease, and ubiquitously expressed calcium-activated intracellular cysteine protease that exists in both cytosol and mitochondria. As a channel protein, calpain 1 expression is closely related to the concentration of calcium ions in neurocytes, cardiomyocytes and other cell types. Calpain-1 activation, resulting from NMDA receptor on postsynaptic membrane, mainly triggered an early neuroprotective signaling cascade potentially in a small subset of neurons in hippocampus, and was restricted to a small population of interneurons following systemic kainic acid injection (Seinfeld et al., 2016). Calpain-1 has also been demonstrated a critical role in synaptic plasticity and learning and memory, as its deletion in mice results in impairment in theta-burst stimulation (TBS)-induced LTP and various forms of learning and memory (Liu Y. et al., 2016). The continuous increase in calpain 1 expression will inevitably lead to excessive calcium influx into cells, resulting in a significant decline of cell survival rate; whereas selective calpain inhibitors have been proved the potency, efficacy and safety as possible therapeutics against abnormal synaptic plasticity and memory produced by the excess of amyloid-β, a distinctive marker of Alzheimer's disease (Fà et al., 2015). Thompson found that the activation of mit-calpain 1 increased cardiac injury during ischemia-reperfusion (IR) by releasing apoptosis-inducing factor, sensitizing mitochondrial permeability transition pore (MPTP) opening, and impairing mitochondrial metabolism through damaging complex I. MDL-28170, an inhibitor of calpain 1, could effectively alleviate cardiac injury during IR by inhibiting both cytosolic and mitochondrial calpain 1 (Thompson et al., 2016). The results of Wang et al. (2016) demonstrated that IH increased calpain enzyme activity and reactive oxygen species (ROS) level as well as Ca^{2+} concentration, and these effects could be eliminated by a membrane-permeable ROS scavenger. Therefore, they insisted that the activation of calpains by ROS-dependent elevation of Ca^{2+} mediate human ether-a-go-go-related gene (hERG) channel protein degradation by IH (Wang et al., 2016). Our investigation showed that the abnormal expression of calpain 1 was related to the ultrastructural damage of hippocampus to some extent during CO exposure. These results were consistent with those of Fà et al. (2015) and Wang et al. (2016), but slightly different from Seinfeld et al. (2016). We conceive the different roles of calpain 1 in the process of pathological state, i.e., the slight and transient increase of calpain 1 expression may play an endogenous protection in the super early stage of CO poisoning, whereas the activation of NMDA receptor and the overload of intracellular calcium will result in the over-expression of calpain 1 protein in a bite late period after exposure to CO, and then lead to cell apoptosis/necrosis through

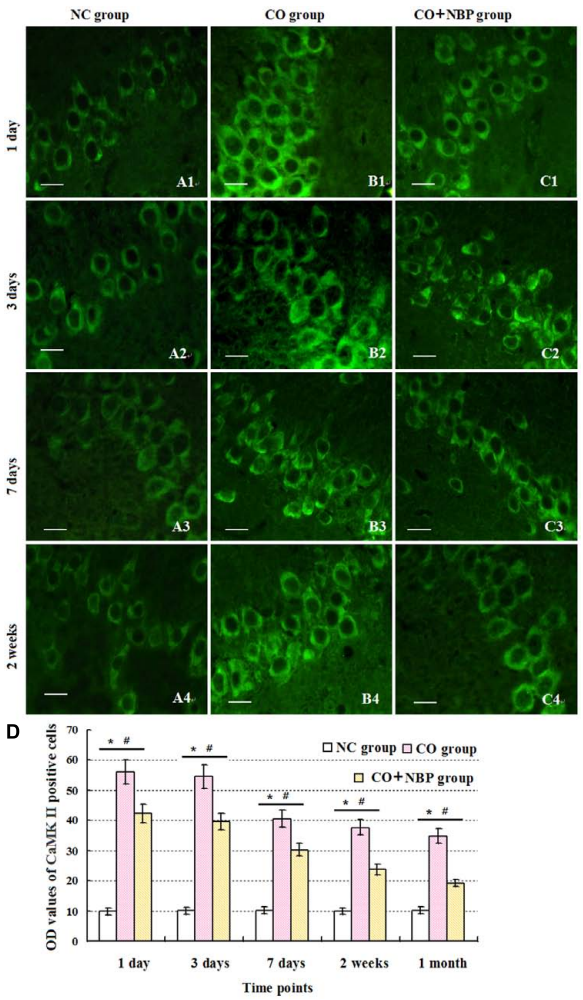

FIGURE 7 | Alterations of CamK II positive cells in each group. CaMK II positive cells were observed in rats in NC group **(A1–A4)**, while the amount of CaMK II positive cells in CO poisoning individuals was sharply increased to the peak between days 1 and 3 with strong fluorescent values, and then decreased **(B1–B4)**. Nevertheless, it was still higher than that of NC group till 1 month ($n = 4$, $P < 0.05$). NBP administration could down-regulate the expression level of CamK II compared with that in CO group at the same times (**C1–C4**; $n = 4$, $P < 0.05$). **(D)** Histogram of the OD values of CaMK II positive cells in each group at different time points. *Compared with NC group ($n = 4$, $P < 0.05$); #compared with CO group ($n = 4$, $P < 0.05$). $F = 10.941 \sim 22.437$. Scale bar is 30 μm.

mitochondrial-mediated signaling pathway (**Figure 9**). NBP treatment could notably decrease the expressions of calpain 1-positive cells, suggesting that NBP may efficiently protect hippocampus neurons against CO toxicity via down-regulating the expression of calpain 1 protein in brain tissue in rats followed by CO poisoning.

Calcium/calmodulin-dependent protein kinase II (CaMK II) is a major multiple functional calcium-regulated enzyme and abundant in brain tissue, especially in hippocampus, which regulates neuronal receptor- gated ion channels, calcium-dependent ion currents and the synthesis and release of neurotransmitters. It has been demonstrated that persistent activation of CaMK II is dependent on the autophosphorylation of Ca^{2+}/calmodulin, and the latter in hippocampus plays a critical role in synapse formation, receptor and ion channel function, gene expression, and memory processing and neuroplasticity. Many experimental animal models revealed that formation of learning and memory, such as hippocampal-dependent spatial learning, is strongly responsible for the activity of CaMK II (Malik and Hodge, 2014). Thus, prevention of CaMK II autophosphorylation could obviously impair spatial learning and memory tasks in mutant mice (Giese et al., 1998), whereas the administration of morphine sensitization apparently increased both Ca^{2+}/calmodulin- independent and -dependent activities of CaMK II in hippocampus in rat models (Kadivar et al., 2014). Moreover, Ashpole and Hudmon (2011) found that a short time suppression of the abnormal CaMK activation could reduce the mortality rate of hippocampal neurons, while the neuroprotective effect was lost, and cell death would inevitably occur if the sustained inhibitory of CaMK expression was more than 8 h, and vice versa. Nevertheless, acute morphine induction at a dosage of 5 mg/kg did not alter either CaMK II mRNA expression or CaMK II activity in hippocampus, and overexpression of CaMK II in transgenic mice resulted in the enhancement of spatial memory acquisition (Mayford et al., 1995). CaMK II, the downstream signal molecular of *N*-methyl-D-aspartate receptors (NMDARs) and cAMP- response element binding protein (CREB), plays a crucial role in inducing the formation of LTP, whose generation and maintenance need the synthesis of new proteins (Zhu et al., 2014). Thus, as a "molecular switch," the relatively invariable activity of CaMK II in cytoplasm may be essential for cell survival and trigger LTP process and short-term memory formation. Our result showed that the level of CaMK II increased sharply during short time intervals, and still maintained at a higher level till 1 month after exposure to CO even up to 1 month. This result was identical to that of calpain 1 expression. Thus, we assumed that under normal circumstances, NMDARs were not activated due to the combination with magnesium ions, the concentration of intracellular calcium was relatively low, and there was only a small amount of calpain 1 and CaMK II proteins in cytoplasm to maintain the structural and functional integrity of cells. However, when suffered a strong or persistent stimulus, such as acute severe CO poisoning, magnesium ions were escaped from the complex and NMDARs were further completely activated. Therefore, the overexpressions of calpain 1 and CaMK II proteins in cytoplasm induced by calcium overload were rush into nucleus, eventually led to degradation of DNA and NPC, and even apoptosis/necrosis (Wang et al., 2013; Chimura et al., 2015; **Figure 9**), whereas early application of NBP can significantly reduce the expressions of calpain 1 and CaMK II proteins, suggesting that NBP may improve cognitive function and maintain neuronal survival and function via inhibiting these two target proteins in rats after CO poisoning.

In summary, the results demonstrated that NBP at the dosage of (6 mg/ 100g) was safe via oral and no side effect was found in any of the SD rats in the present study. NBP

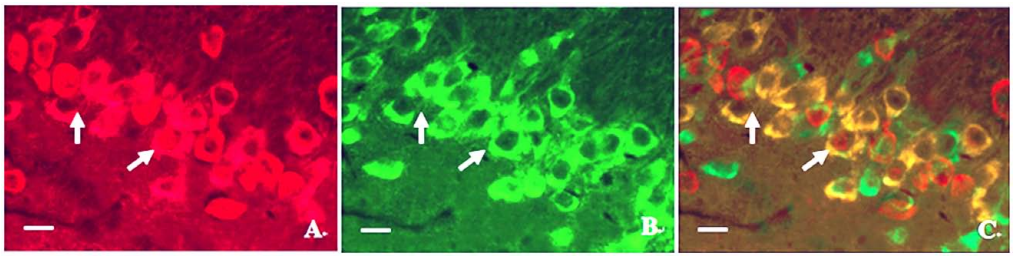

FIGURE 8 | Photographs of relationship between the locations of calpain 1 and CaMK II proteins in hippocampus under a fluorescent microscope. **(A)** Calpain 1 positive cells; **(B)** CaMK II positive cells; **(C)** co-expressions of the two proteins under the same view (merged). Scale bar is 30 μm.

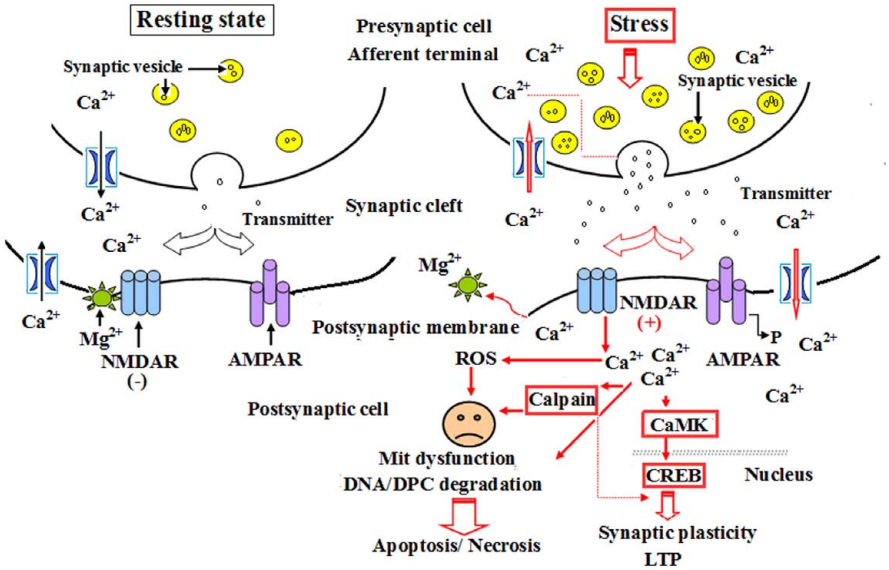

FIGURE 9 | A schematic diagram of the role of calpain 1 and CaMK II in apoptosis and long-term potentiation (LTP) after CO poisoning. Under resting state conditions, NMDARs were interacted with magnesium ion and were not activated, the level of intracellular calcium ions was relatively low, and there was only a small amount of calpain 1 and CaMK II proteins in cytoplasm to maintain the structural and functional integrity of cells. Under the pathological circumstances, such as a strong or persistent electrical stimulation, ischemia and hypoxia, acute severe CO poisoning, NMDARs were completely activated, and the overexpressions of calpain 1 and CaMK II proteins in cytoplasm induced by calcium overload were rush into nucleus, thus participated in the process of LTP and apoptosis or necrosis.

treatment can efficiently improve learning and memory function, maintain the structure integrity of hippocampal neurons, inhibit the expressions of memory-related proteins, thereby preventing rats from cognitive impairment after exposure to CO. The neuroprotective effect of NBP is involved in the down-regulation of both calpain 1 and CaMK II expression. Early application of NBP may be more conducive to the resumption of cognitive function in patients with acute CO poisoning.

CONCLUSION

Based on these findings, we inferred that NBP treatment could improve the ultrastructure and cognitive function of hippocampus in rats with CO poisoning, which is associated with the down-regulation of both calpain 1 and CaMK II proteins.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: QL and D-DG. Performed the experiments: X-NS, X-YD, Y-HZ, and BL. Analyzed the data: M-JB and YZ. Contributed reagents/materials/analysis tools: M-JB. Wrote the paper: QL and D-DG.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (NO: 81571283), the Traditional Chinese Medicine Science and Technology Development Project in Shandong (NO: 2015-420) and the Medical and Health Development Project Grants in Shandong (NO: 2014WS0248). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer YG declared a shared affiliation, though no other collaboration, with the authors to the handling Editor, who ensured that the process nevertheless met the standards of a fair and objective review.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 27 February 2017
doi: 10.3389/fphar.2017.00093

Cannabidiol Affects MK-801-Induced Changes in the PPI Learned Response of Capuchin Monkeys (*Sapajus* spp.)

*Patricia G. Saletti[1], Rafael S. Maior[1,2], Marilia Barros[3], Hisao Nishijo[4] and Carlos Tomaz[1,5]**

[1] Primate Center and Laboratory of Neurosciences and Behavior, Department of Physiological Sciences, Institute of Biology, University of Brasilia, Brasilia, Brazil, [2] Department of Clinical Neuroscience, Psychiatry Section, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden, [3] Department of Pharmaceutical Sciences, School of Health Sciences, University of Brasilia, Brasilia, Brazil, [4] System Emotional Science, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan, [5] Neuroscience Research Group, University CEUMA, São Luís, Brazil

There are several lines of evidence indicating a possible therapeutic action of cannabidiol (CBD) in schizophrenia treatment. Studies with rodents have demonstrated that CBD reverses MK-801 effects in prepulse inhibition (PPI) disruption, which may indicate that CBD acts by improving sensorimotor gating deficits. In the present study, we investigated the effects of CBD on a PPI learned response of capuchin monkeys (*Sapajus* spp.). A total of seven monkeys were employed in this study. In Experiment 1, we evaluated the CBD (doses of 15, 30, 60 mg/kg, i.p.) effects on PPI. In Experiment 2, the effects of sub-chronic MK-801 (0.02 mg/kg, i.m.) on PPI were challenged by a CBD pre-treatment. No changes in PPI response were observed after CBD-alone administration. However, MK-801 increased the PPI response of our animals. CBD pre-treatment blocked the PPI increase induced by MK-801. Our findings suggest that CBD's reversal of the MK-801 effects on PPI is unlikely to stem from a direct involvement on sensorimotor mechanisms, but may possibly reflect its anxiolytic properties.

Keywords: cannabidiol (CBD), MK-801, monkey, prepulse inhibition, learning, memory

OPEN ACCESS

Edited by:
Arjan Blokland,
Maastricht University, Netherlands

Reviewed by:
Glenn W. Stevenson,
University of New England, USA
Ledia F. Hernandez,
CINAC and Hospital Universitario HM Puerta Del Sur, Spain

***Correspondence:**
Carlos Tomaz
ctomaz@ceuma.br

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *05 December 2016*
Accepted: *13 February 2017*
Published: *27 February 2017*

Citation:
Saletti PG, Maior RS, Barros M, Nishijo H and Tomaz C (2017) Cannabidiol Affects MK-801-Induced Changes in the PPI Learned Response of Capuchin Monkeys (Sapajus spp.). Front. Pharmacol. 8:93. doi: 10.3389/fphar.2017.00093

INTRODUCTION

Cannabidiol (CBD) is one of the several compounds extracted from the Cannabis plant reported to have different therapeutic applications (Baker et al., 2003). These seem to include general systemic anti-inflammatory (Mechoulam et al., 2002; Vilela et al., 2015) and antioxidant properties (Hampson et al., 1998), as well as more specific effects in different neurological disorders, such as epilepsy (Mechoulam et al., 2002), anxiety (Zuardi et al., 1982; Campos and Guimarães, 2008), and schizophrenia (Zuardi et al., 1991; Leweke et al., 2012).

The antipsychotic effects, in particular, may result from the antagonistic action of CBD on cannabinoid type 1 (CB1) and 2 receptors (CB2; Thomas et al., 2007). Zuardi et al. (2012) suggested, however, that an interaction between CBD and anandamide is essential for the psychoactive effect, considering that CBD also increases the availability of this endocannabinoid via a reuptake inhibition mechanism (Mechoulam et al., 2002). The antipsychotic properties of CBD have also been linked to changes in glutamate signaling that results from the activation of the vanilloid TRPV type 1 receptor system. Anandamide (Di Marzo et al., 2001) and CBD (Cristino et al., 2006) are both known TRPV1 receptor ligands, and the pre-synaptic activation of this

system increases the release of glutamate in psychosis-related areas of the brain (Xing and Li, 2007). Furthermore, CBD is reported to reverse the prepulse inhibition (PPI) disruption induced by the non-competitive glutamate NMDA receptor antagonist dizocilpine (MK-801) in murine models (Long et al., 2006; Gomes et al., 2014; Levin et al., 2014; however, see Gururajan et al., 2011), with this effect being antagonized by the TRPV1 receptor antagonist capsazepine (Long et al., 2006).

In fact, MK-801 has become a frequently used pharmacological tool to induce schizophrenic-like symptoms in pre-clinical experimental setups (Arai et al., 2008; Park et al., 2014; Saletti et al., 2015). Schizophrenic patients commonly demonstrate significant deficits in sensorimotor gating (Braff et al., 2001). This dissociative anesthetic is also reported to disrupt the PPI responding of rodents (Hoffman et al., 1993), as well as to induce cognitive (Hikichi et al., 2013; Karamihalev et al., 2014) and social recognition deficits (Yoshimi et al., 2015) and hyperlocomotion (Park et al., 2014; Basurto et al., 2015). However, to our knowledge, the effects of CBD on the PPI response of non-human primates (NHP) have yet to be assessed. It is known that manipulations of learning and memory-related areas, such as the hippocampus, can alter PPI responding (Kohl et al., 2013). Compared to rodents, NHPs have a distinct motor response to cannabinoid-related substances (Meschler et al., 2001) and display higher CB1 receptor densities in memory-related areas (Ong and Mackie, 1999). So, here we first analyzed the effects of CBD directly on the PPI response of capuchin monkeys and then evaluated the influence of a CBD pre-treatment on repeated MK-801-induced changes in the same PPI test.

MATERIALS AND METHODS

Ethics Statement

All the procedures herein complied with the Brazilian regulations for the scientific use of laboratory animals (Lei Arouca 11.794/2008), as well as the CONCEA/Brazil and NIH/USA guidelines for the care and use of laboratory animals, and were approved by the Animal Ethics Committee of the University of Brasilia (no. 131791/2013).

Subjects and General Housing Conditions

In total, seven capuchin monkeys (*Sapajus* spp.) were used, one male and six females, weighing between 2.5 and 5.0 kg at the beginning of the study. All subjects were housed and tested at the Primate Center of the University of Brasilia, Brazil. They were group or paired-housed under natural light, temperature and humidity conditions in standard cages (3 m × 3 m × 1.8 m) containing rope swings, nest boxes and natural substrate on the floor. Fresh food was provided daily at 07:00 h, consisting of a mixture of pieces of fruits and vegetables. Boiled eggs, nuts and cooked chicken breast were given several times a week, also at 07:00 h. Unconsumed items were removed at 17:00 h, while water and chow were available *ad libitum*.

Housing and maintenance conditions complied with the regulations of the Brazilian National Institute of Environment and Renewable Natural Resources (IBAMA). The animals were only deprived of food and water during the specific trials indicated in the procedure below. All subjects used had previous experience with the PPI protocol (Saletti et al., 2014).

Startle Measurement and General Procedure

Prepulse inhibition testing was performed in a transparent acrylic chamber (60 cm × 30 cm × 30 cm) placed above a wooden box (45 cm × 40 cm × 40 cm). Each subject was placed inside this chamber in such a way that its head protruded through an adjustable central neck hole located at the top. A transparent acrylic device (30 cm × 30 cm × 25 cm) was placed directly on top of the chamber, encompassing the subject's head. This device contained three speakers (model FT96H- frequency band 4 KHz∼30 KHz; Fostex, Japan), each located 10 cm from the monkey's head and connected to a sound generator (O'Hara & Co., Ltd., Japan). One speaker was positioned on each side of the animal's head and generated the startle stimuli. The third speaker was placed at the back of the device and emitted a constant 65dB white noise. An accelerometer (model BDK3; Inntechno Japan Co.Ltd., Japan), connected to an amplifier (O'Hara & Co., Ltd., Japan), was placed at the bottom of the chamber. When the subject was inside the chamber it stood on the accelerometer platform. The accelerometer captured the animal's whole-body movement and transmitted the data to be recorded on the Animal Startle software (PCI 6024E, developed by O'Hara & Co., Ltd., Japan) that interfaced with the Windows XP system (for details see Saletti et al., 2014). A video camera (model #1004124; Clone, Brazil) was connected to this experimental setup and monitored the animal during each session.

Testing was conducted 5 days a week, between 8:00 and 12:00 h, in an acoustically isolated room located near the monkeys' home-cage. After a 10 min acclimatization period to the test room and setup described above, PPI testing was conducted. It consisted of 10 consecutive blocks of stimuli presentation, held at 60 s intervals. During each block, three different stimuli were randomly presented, also at 60 s intervals: a 115 dB pulse of 40 ms duration, an 80 dB prepulse of 20 ms duration and a prepulse-pulse combination with a 120 ms interval between the prepulse and pulse presentation. Stimuli intensity and duration were based on previous studies (Saletti et al., 2014, 2015). The startle response was recorded by the Animal Startle software system that measured the animals' body movements through changes in force detected by the accelerometer. The animals' body movements were recorded during a 600 ms post-stimuli interval and the peak amplitude registered following the pulse or prepulse+pulse stimulus of each trial was recorded as the startle amplitude.

Experiment 1: Effects of CBD Treatment on PPI

In the first experiment, CBD (0, 15, 30, and 60 mg/kg; volume of 1 mL/kg; STI Pharm, UK) was dissolved in a 1:19 solution of

Tween 80 (Sigma-Aldrich, Brazil) and 0.9% saline, respectively. Doses were based on previous studies in rodents (Moreira and Guimarães, 2005; Almeida et al., 2013; Levin et al., 2014). For the safety of the personnel involved and to insure correct intraperitoneal (ip) administrations, all animals were briefly exposed to the anesthetic isoflurane via inhalation, yet an effective anesthesia stage was never really attained.

All subjects were initially tested with vehicle and then treated with the three doses of CBD. Only one treatment was given on each test day, with a 2-week interval between trials. The order in which the three CBD doses were given was randomly assigned for each animal. Each subject received the vehicle injection or a CDB dose and was then placed in the test chamber. After a 30 min interval, it was submitted to the PPI test procedure described above. Two females were excluded from the analyses due to data recording problems, totalizing five animals in experiment 1 (one male and four females).

Experiment 2: Effects of CBD Pre-treatment Following Repeated MK-801 Treatment

In this second experiment, the same seven monkeys were tested 4 months after the previous study. MK-801 (0.02 mg/kg; Sigma-Aldrich, Brazil) and CBD (60 mg/kg; STI Pharm, UK) were both dissolved in a 1:19 solution of Tween 80 (Sigma-Aldrich, Brazil) and 0.9% saline, respectively. The former was administered intramuscularly (im), whereas CBD was again given via ip route. The injection volume of both substances was 1 ml/kg. The MK-801 dose was based on our previous study (Saletti et al., 2015).

Each animal was given a MK-801 injection, once a week, during three consecutive weeks. Twenty minutes after each injection, the subject was tested in the same PPI procedure described above. On the 4th week of testing, each monkey was pre-treated with CBD and then 10 min later it received a MK-801 treatment. PPI testing was once again held 20 min after the MK-801 administration. Two weeks later, each animal was injected with vehicle and following a 20 min interval tested in the PPI protocol (VEH 2). To control for a habituation effect due to repeated testing, and considering that the same monkeys were used in both experiments, data from the vehicle trial of Experiment 1 were used as a second control session in this study (VEH 1).

Behavioral and Statistical Analysis

Data were expressed as the mean startle response or percentage of PPI + standard error of the mean (+S.E.M.). The data were normalized using the following calculation of the percentage of inhibition (Saletti et al., 2014, 2015):

$$\frac{100*(p - pp)}{p} \quad (1)$$

where p corresponds to the pulse-alone startle response and pp to the prepulse+pulse response.

The data were normally distributed, according to the Shapiro–Wilk test. To establish a possible between-treatment effect (vehicle, MK-801 and CBD+MK-801) on the capuchins' startle amplitude and percentage of PPI, data from each experiment were analyzed using a one-way ANOVA for repeated measures (pulse and prepulse+pulse trials were analyzed separately). Whenever significant results were obtained, Fisher's LSD *post hoc* test was used for pair-wise comparison across treatments. Significance level for all tests was set at $p < 0.05$. For Shapiro–Wilk and ANOVA analysis we used the IBM SPSS® version 20 software. For comparisons with $p < 0.05$, we also calculated the effect sizes (effsize) using Matlab software® (version R2013a).

RESULTS

Experiment 1: Effects of CBD Treatment on PPI

In terms of the startle amplitude, a significant between-treatment effect was not observed for either the pulse ($F_{3,12} = 0.566$, $p = 0.648$; **Figure 1A**) or prepulse+pulse trials ($F_{3,12} = 1{,}375$;

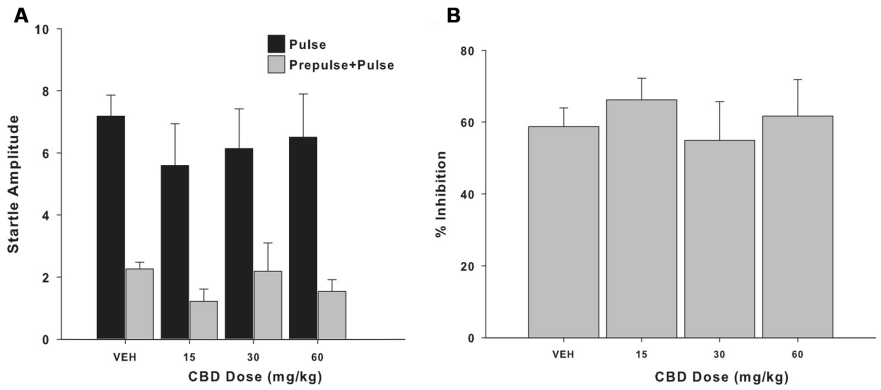

FIGURE 1 | (A) Mean (+SEM) startle amplitude of the capuchin monkeys ($n = 5$) in the pulse (black bars) and prepulse-pulse trials (gray bars) following the vehicle (VEH) and the three CBD treatments (15, 30, and 60 mg/kg). **(B)** Mean (+SEM) percentage of prepulse inhibition (PPI) of the capuchin monkeys ($n = 5$) following the vehicle (VEH) and the three CBD treatments (15, 30, and 60 mg/kg).

$p = 0.297$; **Figure 1A**), with the subjects' response remaining constant regardless of the treatment received. A similar profile was seen for the percentage of PPI when analyzing the data according to the treatment received ($F_{3,12} = 0.368$, $p = 0.778$; **Figure 1B**) and over the course of the weeks of the experiment, regardless of the specific treatment received ($F_{3,12} = 1.283$, $p = 0.325$; **Figure 2**).

Experiment 2: Effects of CBD Pre-treatment on MK-801-Induced Changes in PPI

Among the different pulse ($F_{5,30} = 1.270$, $p = 0.303$; **Figure 3**) or prepulse+pulse trials ($F_{5,30} = 1.800$, $p = 0.143$; **Figure 3**), significant differences were not observed. However, the percentage of PPI differed significantly between treatments ($F_{5,30} = 4.052$, $p = 0.006$; **Figure 4**). This parameter was significantly higher following all three MK-801 administrations, compared to the first vehicle administration [vs. MK-801(1): $p = 0.009$, effsize $= 1.498$; vs. MK-801(2): $p = 0.026$, effsize $= 0.983$; vs. MK-801(3): $p = 0.005$, effsize $= 1.743$]. The percentage of PPI after the CDB+MK-801 treatment was significantly lower than on the trials with only MK-801(1) and MK-801(3), being similar to the levels seen following both vehicle injections [vs. VEH 1: $p = 0.183$; vs. MK-801(1): $p = 0.02$, effsize $= 0.718$; vs. MK-801(2): $p = 0.512$; vs. MK-801(3): $p = 0.013$, effsize $= 0.878$; vs. VEH 2: $p = 0.954$].

DISCUSSION

CBD on Monkey PPI

The results from Experiment 1 indicated that the CBD administration alone had no effect on the capuchin monkeys' startle amplitude or PPI response. Although to our knowledge this is the first study to evaluate such aspect in NHPs, similar results have been observed in rodents (Long et al., 2006; Gomes et al., 2014; Levin et al., 2014; Pedrazzi et al., 2015). Long et al. (2006) and Gururajan et al. (2011) reported, however, that a systemic administration of CBD altered the startle response of rodents. In this case, the percentage of PPI decreased following a 10 mg/kg dose of CBD, whereas 3 and 30 mg/kg had no effect.

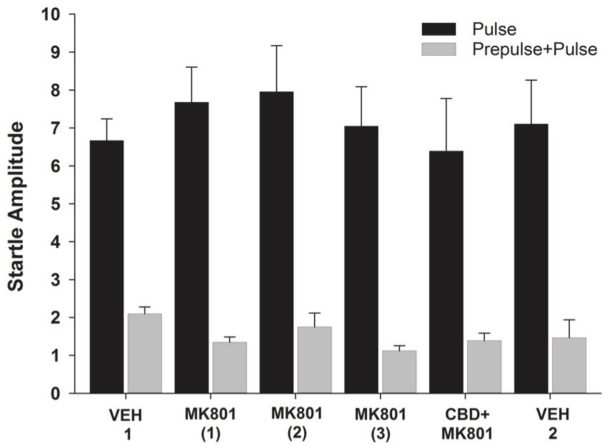

FIGURE 3 | Mean (+SEM) startle amplitude of the capuchin monkeys ($n = 7$) in the pulse (black bars) and prepulse-pulse trials (gray bars) after vehicle administration (VEH 1), the injections of MK-801 (1–3) held once a week during three consecutive weeks, the CDB pre-treatment followed by a MK-801 treatment (CBD+MK801) held on the 4th week, and the second vehicle administration (VEH 2) performed on the 6th week. Except for VEH 1, held during Experiment 1, all other data were recorded during Experiment 2.

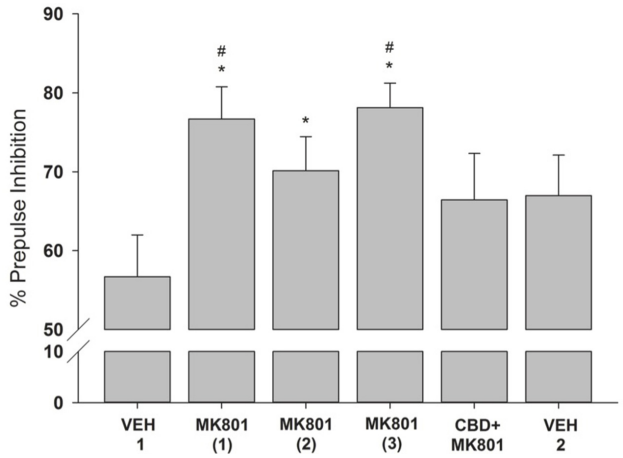

FIGURE 4 | Mean (+SEM) percentage of PPI of the capuchin monkeys ($n = 7$) following the vehicle administration (VEH 1), the injections of MK-801 (1–3) held once a week during three consecutive weeks, the CDB pre-treatment followed by a MK-801 treatment (CBD+MK801) held on the 4th week, and the second vehicle administration (VEH 2) performed on the 6th week. Except for VEH 1, held during Experiment 1, all other data were obtained during Experiment 2. *$p < 0.05$ vs. VEH 1; #$p < 0.05$ vs. CBD+MK-801.

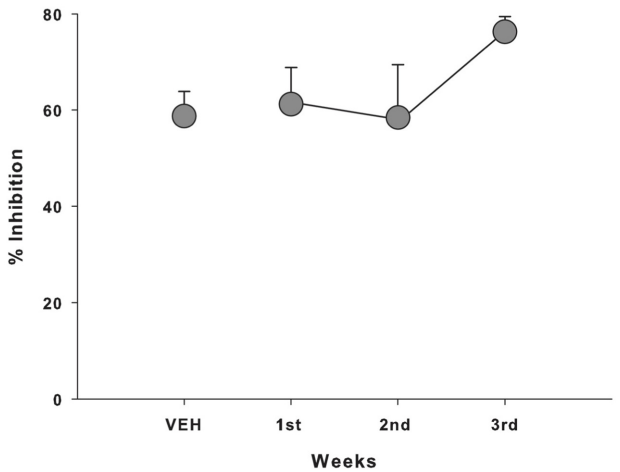

FIGURE 2 | Mean (±SEM) percentage of PPI of the capuchin monkeys ($n = 5$) according to the week of Experiment 1, regardless of the CBD dose received; VEH, vehicle.

This discrepancy may be partly due to species-specific differences, as the CB1 receptor density of rodents and primates differs in certain regions of the brain (Ong and Mackie, 1999), as well as their motor response to cannabinoid-related substances (Meschler et al., 2001). Important methodological aspects may also have contributed. Due to ethical restrictions and reduced sample size in monkey research, our subjects were submitted to the PPI procedure in a repeated-exposure design. Nonetheless, a temporal or drug-training effect does not seem to have influenced the present result as the percentage of PPI remained constant over the course of the procedure, regardless of the specific CBD treatment given. Thus, our results in capuchins suggest that CB1 receptor antagonism may have no effect on sensorimotor gating mechanisms.

CBD Pre-treatment on MK-801 Induced PPI Enhancement

MK-801 has been shown to induce schizophrenic-like behaviors in monkeys, such as deficits in memory-related processing (Ogura and Aigner, 1993; Buffalo et al., 1994; Harder et al., 1998; Harder and Ridley, 2000; Tsukada et al., 2005; Wang et al., 2012) and more recently in changes in PPI responding (Saletti et al., 2015). Although an acute 0.03 mg/kg dose of MK-801 decreased the PPI of capuchin monkeys, repeated testing (with different doses) in the same setup diminished this effect possibly due to drug-induced ataxia or tolerance (Saletti et al., 2015).

In the present study, we opted to use a lower dose of MK-801 to reduce such levels of ataxia. The 0.02 mg/kg dose was used in a repeated administration regimen, thereby leading to an increase in the monkeys' percentage of PPI. This result differs from the MK-801-induced decrease in PPI reported in rodents (Schulz et al., 2001; Gomes et al., 2014), considered to be an indicator of a psychotic-like effect of this drug. It also contrasts with the lack of effect observed for this dose in our previous study using the same animals (Saletti et al., 2015). In that instance, each subject received all possible treatments only once (vehicle, 0.01, 0.02, and 0.03 mg/kg) in a pseudo-randomized order, whereas only the 0.02 mg/kg dose was presently given on three consecutive trials. Thus the present increase in PPI, detected already on the first trial, seems unlikely to be due to a habituation effect considering the 5-month interval between the experiments, the similar PPI and startle amplitude levels seen during the vehicle control trials of the two studies (≈55% and 6 points, respectively), and the response stability seen on the last session of current study (VEH 2 vs. CBD+MK801). Ketamine, another NMDA antagonist, is also reported to increase the PPI response of healthy human volunteers when using a brief inter-stimulus interval (Braff et al., 2001; Duncan et al., 2001). The use of higher doses in rodents and distinct hippocampal NMDA receptor sensitivity could be important aspects contributing to the discrepancies between human and rodent PPI responding (Duncan et al., 2001). NMDA antagonists can block hippocampal long-term potentiation, with this effect being modified by pre-training or test familiarization through latent learning effect (Otnaess et al., 1999; Ennaceur et al., 2011). The hippocampus is also known to be responsable for PPI response modulation (Kohl et al., 2013).

Interestingly, pre-treatment with CBD prevented the MK-801-induced increase in the monkeys' PPI. Considering the large effect sizes values found (≈0.8 or more; Sullivan and Feinn, 2012), we are able to conclude that the difference between the treatments is expressive. Nonetheless, the effects induced by both MK-801 and CBD seem related to their anxiogenic and anxiolytic profiles, respectively – an aspect that is highly relevant in the context of a startle response. In mice, MK-801 is reported to increase anxiety levels (Ennaceur et al., 2011), whereas CBD has been frequently linked to anxiolysis (Zuardi et al., 1982; Guimarães et al., 1990; Campos and Guimarães, 2008; Almeida et al., 2013). CBD seems to activate serotoninergic 5-HT1A receptors (Russo et al., 2005; Resstel et al., 2009; Soares et al., 2010; Gomes et al., 2011) and attenuate cardiovascular and/or behavioral responses associated with anxiety and panic in rats (Resstel et al., 2009; Soares et al., 2010).

Although CBD has been reported to reverse the PPI disruption induced by MK-801 (Long et al., 2006; Gomes et al., 2014) and amphetamine in rodents (Pedrazzi et al., 2015), our present results with capuchins fail to corroborate a possible anti-psychotic effect for this compound in the context of sensorimotor gating. In the presence of only MK-801, the startle amplitude generally tended to decrease in the 'pulse' trials, yet increase in 'prepulse+pulse' trials. The latter, however, was also observed with subsequent treatments (CBD+MK-801 and VEH 2). The pre-treatment of the monkeys with CBD reversed this increase tendency in startle amplitude in PPI, with no visible changes in 'pulse' trials. Altogether, these results suggest that the CBD effects on PPI are unlikely to stem from a direct involvement in sensorimotor gating mechanisms. We suggest that the CBD-induced reversal of the MK-801 effects may be related to their anxiolytic and anxiogenic profiles, respectively (Zuardi et al., 1982; Ennaceur et al., 2011; Almeida et al., 2013), although this aspect was not directly investigated in the present study. Further studies are necessary to better elucidate the interplay between anxiety and schizophrenia, as well as the potential use of CBD as an antipsychotic drug, possibly using other NHP models as well.

CONCLUSION

In the present study, CBD alone had no effect on the PPI response of capuchin monkeys, yet blocked the increase in this response that was induced by NMDA receptor antagonism (MK-801). This effect may stem from a general anxiolytic rather than an anti-psychotic effect, corroborating an anxiolytic profile of CBD in the PPI paradigm.

AUTHOR CONTRIBUTIONS

CT, PS, and RM: Conception and design, acquisition of data, or analysis and interpretation of data; drafting the article or

revising it critically for important intellectual content. MB and HN: Drafting the article or revising it critically for important intellectual content.

FUNDING

This research was supported by FAP/DF grant to CT (no. 193.000.033/2012) and the SPS Asian Core Program. PS was recipient of a doctoral fellowship from CNPq, MB a research fellowship from CNPq (304041/2015-7) and RM a post-doctoral fellowship from CNPq (233647/2014-7).

ACKNOWLEDGMENT

We thank G. V. da Silva, A. Araujo, R. S. Oliveira, and C. D. Azevedo for the excellent animal care.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 21 June 2017
doi: 10.3389/fphar.2017.00395

Scopolamine Induces Deficits in Spontaneous Object-Location Recognition and Fear-Learning in Marmoset Monkeys

*Jonathan L. Melamed[1], Fernando M. de Jesus[2], Rafael S. Maior[2] and Marilia Barros[1]**

[1] Department of Pharmaceutical Sciences, School of Health Sciences, University of Brasilia, Brasilia, Brazil, [2] Primate Center and Department of Physiological Sciences, Institute of Biology, University of Brasilia, Brasilia, Brazil

The non-selective muscarinic receptor antagonist scopolamine (SCP) induces memory deficits in both animals and humans. However, few studies have assessed the effects of amnesic agents on memory functions of marmosets – a small-bodied neotropical primate that is becoming increasingly used as a translational model for several neuropathologies. Here we assessed the effects of an acute SCP administration (0.03 mg/kg, sc) on the behavior of adult marmoset monkeys in two tasks. In the spontaneous object-location (SOL) recognition task, two identical neutral stimuli were explored on the sample trial, after which preferential exploration of the displaced versus the stationary object was analyzed on the test trial. In the fear-motivated behavior (FMB) procedure, the same subjects were submitted to an initial baseline trial, followed by an exposure period to a snake model and lastly a post-exposure trial. All trials and inter-trial intervals lasted 10 min for both tests. Results showed that on the SOL test trial, the saline group explored the displaced object significantly longer than its identical stationary counterpart, whereas SCP-treated marmosets explored both objects equivalently. In the FMB test, the saline group – but not the SCP-treated animals – spent significantly less time where the stimulus had been specifically encountered and more time being vigilant of their surroundings, compared to pre-exposure levels. Drug-related effects on general activity, overall exploration (SOL task) and behavioral response to the aversive stimulus (FMB task) were not observed. SCP thus impaired the marmosets' short-term ability to detect changes associated with the spatial location of ethologically irrelevant (SOL task) and relevant stimuli (FMB task). Similar results have been reported in other animal species. Marmosets may thus help reduce the translational gap between pre-clinical studies and memory-associated human pathologies.

Keywords: marmoset, object location, recognition, snake, fear memory, scopolamine

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Alexander Easton, Durham University, United Kingdom
Robert Warren Gould, Vanderbilt University, United States

***Correspondence:**
Marilia Barros
mbarros@unb.br

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *13 April 2017*
Accepted: *06 June 2017*
Published: *21 June 2017*

Citation:
Melamed JL, de Jesus FM, Maior RS and Barros M (2017) Scopolamine Induces Deficits in Spontaneous Object-Location Recognition and Fear-Learning in Marmoset Monkeys. Front. Pharmacol. 8:395. doi: 10.3389/fphar.2017.00395

INTRODUCTION

Over the years central cholinergic signaling has become increasingly implicated in different learning and memory processes (Hasselmo and Sarter, 2011). In fact, the loss of specific basal forebrain cholinergic input to the cortex is one of the pathogenic hallmarks of Alzheimer's dementia (Bartus, 2000), with a concomitant decline in cortical choline acetyltransferase (ChAT) activity

also being correlated with cognitive dysfunction in different human pathologies (e.g., dementias, Parkinson disease, brain damage; Candy et al., 1983). In rodents and non-human primates (NHPs), the use of excitotoxic (e.g., *rodents*: Baxter and Bucci, 2013; *marmosets*: Ridley et al., 1986; *macaques*: Aigner et al., 1991a) and more specific immunotoxic lesions (e.g., *rodents*: Easton et al., 2011; *marmosets*: Ridley et al., 1999; *macaques*: Turchi et al., 2005) of basal forebrain cholinergic projections to the cortex disrupted several learning and memory processes. When using this approach, the degree of the impairment can vary significantly according to the specificity and extent of the lesion and the type of cognitive task being assessed, with the possible involvement of non-cholinergic afferents. However, recent optogenetic-based studies have provided compelling evidence in mice for a causal role of basal forebrain cholinergic activity during visual discrimination tasks (Pinto et al., 2013).

The acetylcholine (ACh) muscarinic receptor blocker scopolamine (SCP) is also reported to disrupt memory processes in humans (Ebert and Kirch, 1998), whereas restoration of transmitter functioning reverses this effect (i.e., cholinesterase inhibitors; Roman and Rogers, 2004). There is also now substantial evidence for its participation in memory-related task performance of both rodents and NHPs (Klinkenberg and Blokland, 2010; Robinson et al., 2011; Baxter and Bucci, 2013), yielding similar results as those seen in lesion studies (Robinson et al., 2011). In fact, SCP has become a frequently used pre-clinical pharmacological tool to assess memory (dys)function (Klinkenberg and Blokland, 2010).

Scopolamine administration, for example, can consistently impair NHPs in delayed nonmatching-to-sample tasks (DNMS) of visual recognition memory (e.g., Ridley et al., 1984b,a; Aigner et al., 1991b). Although this task exploits their spontaneous preference for novelty over familiarity, it requires pre-training the monkey to learn response-reward associations and the nonmatching to sample rule. Rodents, on the other hand, are typically assessed in a simpler procedure requiring no prior training or response reinforcement – the one-trial spontaneous object recognition task and its several close variations (Dere et al., 2007). Granted that this procedure also exploits their novelty preference, its basis is the spontaneous explorative behavior displayed during a choice trial that occurs after an initial familiarization period. When treated with SCP, rodents become unable to recognize familiar objects (reviewed in Dere et al., 2007) or their associated spatial locations (Murai et al., 2007; Pitsikas, 2007; Barker and Warburton, 2009; Schäble et al., 2012). Originally tested in rats by Ennaceur and Delacour (1988), spontaneous recognition tasks have since been extended to other animals (e.g., *mice*: Dere et al., 2005; *dogs*: Callahan et al., 2000; *pigs*: Kornum et al., 2007), but to the best of our knowledge still remain to be assessed in NHPs. The ability to recognize whether an object has been encountered in the past is an important element of our declarative memory and a function that becomes impaired, for example, in patients with Alzheimer's disease (Purdy et al., 2002) or who have sustained brain injury (Reed and Squire, 1997).

Cholinergic signaling also seems to play an important modulatory role on fear memories, a type of associative learning that has a high adaptive function against real and potential threats (Tinsley et al., 2004). For instance, during contextual conditioning, a neutral spatial location will come to evoke fear-related behaviors after being associated with an inherently fearful stimulus (Maren et al., 2013). In rodents, fear-conditioned stimuli increased central ACh release (Acquas et al., 1996), whereas SCP-treated animals performed poorly in conditioning tasks (reviewed in Robinson et al., 2011; Wilson and Fadel, 2017). Research on fear memory in NHPs, however, has focused mainly on elucidating the neuronal circuits involved in specific behavioral tasks (*fear-potentiated startle*: Antoniadis et al., 2009; *passive avoidance*: Machado et al., 2009; *cue-conditioning*: Agustín-Pavón et al., 2012). As fear memory processes seem to be altered in several psychopathologies (i.e., posttraumatic stress disorder and schizophrenia; Maren et al., 2013), as well as Alzheimer's disease and other related dementias (Hoefer et al., 2008), new pharmacological-based studies in NHPs may contribute to our current understanding on the neurochemical aspects of learned fear.

The present experiments were thus designed to assess – in both the presence and absence of an acute SCP administration – the behavioral response of adult marmoset monkeys in a spatial recognition memory task and a fear-motivated learning procedure. The marmoset is a small-bodied, diurnal and arboreal neotropical primate. Compared to most NHPs they have a rapid reproductive turnover, shorter life-span, are easily captured and handled, readily adapt to captive conditions and have lower husbandry costs (reviewed in Barros and Tomaz, 2002). These characteristics, along with the recent sequencing of the common marmoset's genome (*Callithrix jacchus*; reviewed in Ward and Vallender, 2012) and development of transgenic individuals (Sasaki et al., 2009) are making these simians an increasingly used translational model of several neuropathologies ('t Hart et al., 2012). In fact, their small lissencephalic brains still retain a large brain-to-body ratio, a well-defined temporal lobe, functional divisions and connectivity of cortical areas, and structure-specific adult neurogenesis similar to those of other anthropoids (e.g., macaques; Stephan et al., 1980; Newman et al., 2009; Burman et al., 2011; Marlatt et al., 2011). Normal adults display the same cytochemical organization of basal forebrain cholinergic neurons of other NHPs and humans, which differs significantly from that of rats (Geula et al., 1993; Wu et al., 2000). Aged marmosets also develop cortical deposits of the beta-amyloid protein typically seen in Alzheimer's dementia patients (Maclean et al., 2000; Geula et al., 2002). Marmosets are capable of performing a variety of memory-related tasks, yet only a few studies have assessed the effects of amnesic agents in marmosets (Ridley et al., 1984b,a; Carey et al., 1992; Harder et al., 1998; Spinelli et al., 2006).

In the first experiment, we used the murine-based one-trial spontaneous object-location (SOL) task (Ennaceur et al., 1997), while in the second experiment contextual fear learning was induced by a snake-related stimulus. Marmosets are highly visually oriented (Forster, 1995), readily attend to spatial cues in their environment (Gaudio and Snowdon, 2008) and react fearfully in response to snakes and related stimuli (Barros et al., 2002).

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of the Brazilian regulations for the scientific use of laboratory animals (Lei Arouca 11.794/2008), as well as the CONCEA/Brazil and NIH/USA guidelines for care and use of laboratory animals. All the procedures herein were approved by the Animal Ethics Committee of the University of Brasilia (no. 33002/2013).

Subjects and Housing Conditions

Nine adult black tufted-ear marmosets were used (*Callithrix penicillata*; 5 males and 4 females), weighing 344 ± 16 g (mean ± SEM; range: 285–460 g) at the beginning of the study. Although the females' estrous cycle was not controlled, none were currently breeding or recently had infants. All subjects were pair-housed at the Primate Center of the University of Brasilia under natural light, temperature and humidity conditions in standard home-cages of a same colony room. Not all cage-mates were included in the present study due to other ongoing experiments. The colony room consisted of two parallel rows of 12 cages each (2 m × 1 m × 2 m; W × L × H), separated by a common wire-mesh enclosed central corridor. A roof covered this central corridor and two-thirds of each home-cage. These were provided with a nest-box, ropes, wood perches, a feeding tray for fresh food and a PVC tube for dry chow. Fresh food was provided daily at 07:30 h, consisting of a mixture of pieces of fruits and vegetables. Boiled eggs, nuts and/or cooked chicken breast were given several times a week, also at 07:30 h. Unconsumed items were removed at 17:30 h. Water and chow were available ad libitum. Housing and maintenance conditions complied with the regulations of the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA).

Apparatus and Experimental Set-up

Testing was conducted in a rectangular open-field (OF) arena (**Figure 1**: 130 cm × 75 cm × 40 cm; W × L × H) suspended 1 m from the floor. Three of its walls were made of aluminum, whereas the fourth was of 4 mm transparent glass. The top consisted of the same glass material and the bottom was made of 2.5 cm^2 wire-mesh. A guillotine-type door on one of the aluminum walls served as the subjects' entry/exit point. With the exception of the glass wall and top, the apparatus was painted white to enhance video-tracking. It was also divided into five quadrants (**Figure 1**): four corner sections of equal dimensions (32.5 cm × 37.5 cm each; W × L) and a larger central zone (65 cm × 75 cm; W × L).

The OF arena was set-up in a test-room located approximately 50 m from the colony facility. The marmosets were transported to and from the test-room in an aluminum transportation cage (35 cm × 20 cm × 23 cm; W × L × H) that attached directly to the arena's door. The apparatus was monitored via a closed-circuit system with two digital cameras (model C920, Logitech, Brazil): one was mounted 1.5 m above the arena and the other was placed 1.5 m in front of its glass wall. Both cameras were connected to a same laptop located in an observation-room adjacent to the test-room. Visual spatial cues were provided by various extra-field items in the test-room.

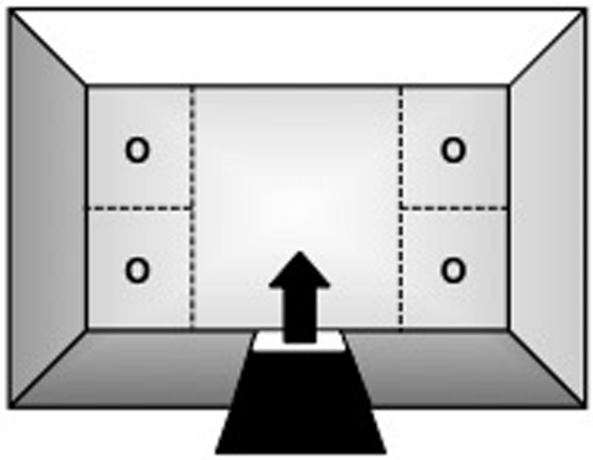

FIGURE 1 | Top-view schematic representation of the marmoset open field apparatus, indicating the subjects' entry/exit point (arrow) via the guillotine-type door, the four corner sections where stimuli could be placed during specific trials of the procedure (O), and the larger central zone.

Drug

Scopolamine hydrobromide (SCP; 0.03 mg/kg; Sigma–Aldrich, United States) was dissolved in phosphate-buffered saline, the latter also being used as vehicle control (SAL). Both substances were injected subcutaneously in a volume of 1.0 mL/kg. The dose and injection-test interval (see Procedure below) were based on previous reports using systemic administrations in marmosets, whereby an inverted U-shaped function was verified (Ridley et al., 1984b: 0.03–1.0 mg/kg; Ridley et al., 1984a: 0.03–0.06 mg/kg; Carey et al., 1992: 0.01–0.04 mg/kg; Harder et al., 1998: 0.06 mg/kg; Spinelli et al., 2006: 0.01–0.06 mg/kg). In these studies, lower doses ranging from 0.02 to 0.06 mg/kg impaired performance in the object discrimination, position discrimination, visuospatial conditional, five-choice serial reaction time and concurrent delayed match-to-position tasks. On the other hand, SCP given at 0.05 or 1.0 mg/kg induced behavioral agitation in marmosets and thus may confound its specific memory effects at higher doses. Based on these studies, we chose to use 0.03 mg/kg as it may selectively disrupt memory, but not other behaviors.

Procedure

All subjects were initially submitted, during three consecutive days, to a daily habituation session that mimicked the general procedure of the subsequent behavioral tasks (see below). Accordingly, each habituation session consisted of an initial 10 min trial, followed by a 10 min inter-trial interval and then a second 10 min trial. The marmoset was given access to the OF apparatus during these two trials of each session, while during the

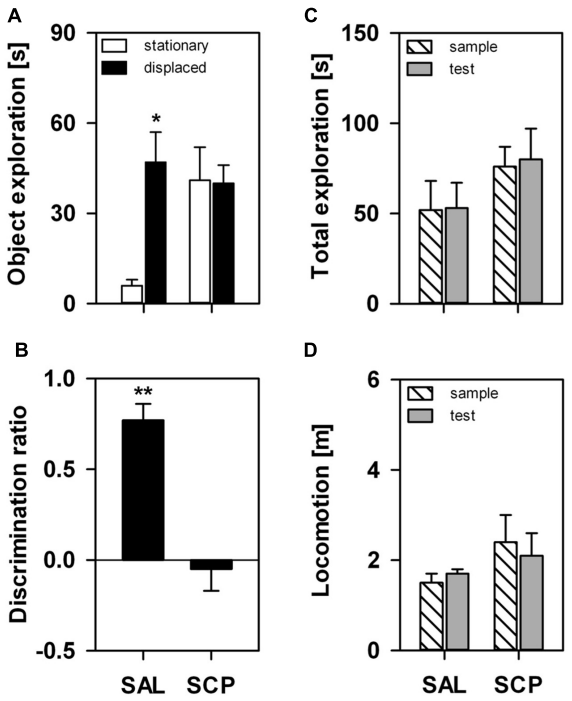

FIGURE 2 | Marmosets' performance on the one-trial *Spontaneous Object-Location (SOL)* Recognition Task. An acute saline (SAL; $n = 4$) or scopolamine (SCP, 0.03 mg/kg SCP; $n = 5$) administration was given subcutaneously 20 min before the task. The sample and test trials lasted 10 min and were held at 10 min intervals. **(A)** Time spent in seconds exploring the stationary and displaced objects on the test trial; **(B)** Object discrimination ratio calculated for the test trial; **(C)** Time spent in seconds exploring both objects on the sample and test trials; and **(D)** Total distance traveled in meters during the sample and test trials. All data are represented as mean + SEM. $^{*}p < 0.05$ vs. the stationary object of the SAL group, $^{**}p < 0.05$ vs. zero-value chance level in the SAL group.

inter-trial interval they were placed in a different holding arena (60 cm × 60 cm × 40 cm; W × L × H) located in the same test-room. The marmosets were transferred between these locations using the transportation cage that attached directly to either arena. The three daily habituation sessions were to familiarize the marmosets with the apparatus and general testing procedure, and thereby no treatment was given and the OF remained empty.

The subjects were then randomly assigned to an experimental group (SCP: $n = 5$ or SAL vehicle: $n = 4$) and individually submitted to the same behavioral tasks described below. On both tasks, the specific location of the objects within the apparatus varied randomly between subjects. The apparatus and objects used were also thoroughly cleaned with a 70% ethanol solution after every trial. All trials were held between 14:00 and 17:00 h.

Spontaneous Object-Location Recognition Task

Based on the murine SOL task (Ennaceur et al., 1997), the marmosets were submitted to a two-trial procedure consisting of an initial 10 min sample trial that was followed, after a 10 min inter-trial interval, by a 10 min test trial. On the sample trial, two identical copies of a small stainless steel bowl (9 cm diameter x 5 cm height) were randomly placed at the center of different corner quadrants of the apparatus and the marmoset was allowed to freely explore the entire arena for 10 min. The objects had not been previously seen by the marmosets, had no apparent ethological significance and could not be displaced by the subjects. After the 10 min retention interval, held in the separate holding arena described above, the subject was again released in the OF for the 10 min test trial. On this trial, two identical copies of the same stainless steel bowl were placed in the arena: one in the same location it had been during the preceding sample trial (stationary object) and the other one in a new position randomly chosen between the previously two unused corner sections (displaced object). The marmoset was again allowed to freely explore the entire arena for 10 min and then returned to its home-cage.

Each subject received its respective treatment 20 min before the start of the SOL task. Systemically administered SCP exerts significant effects on central neuronal function 30 min post-injection (Ebert et al., 2001) and only pre-training SCP treatment has been found to impair SOL recognition memory in rodents (Barker and Warburton, 2009).

Fear-Motivated Behavior (FMB) Test

After a 2-week interval, the same two groups of marmosets were submitted to a three-trial procedure. First, a 10 min baseline pre-exposure trial was held in the OF arena in the absence of any stimulus. After a 10 min inter-trial interval held in the same holding arena, the subject was again released in the apparatus for a 10 min snake exposure trial. For this, a coiled and motionless red-black-white rubber snake model (120 cm long× 2 cm girth) was placed in one of the corner quadrants of the apparatus. As a general preference for any of the corner sections of the OF arena was not observed during the initial baseline trial, the snake model was randomly placed in any one of these locations. The subjects were all snake-naive and unable to displaced this aversive stimulus, which in turn could be seen from any point in the arena. After a second 10 min inter-trial interval held in the holding arena, the marmoset was placed for the third time in the OF apparatus for a 10 min post-exposure trial, in the absence of any stimulus, and thereafter returned to its home-cage.

Each subject received its respective treatment immediately before the start of the initial baseline trial. A snake model was used as an aversive stimulus since NHPs invariably regard them as a potential threatening stimulus (Isbell, 2006). Both feral (Teixeira et al., 2016) and captive marmosets (Barros et al., 2002) promptly react to snakes and related stimuli.

Behavioral Analyses

We used the AnyMaze software (Stoelting Co., United States) to record and analyze the marmosets' behavioral response during each experimental trial. Via the top-view camera, the software automatically tracked the animals' total distance traveled and the time spent in each quadrant of the apparatus. In addition, using the same program and the side-view camera, an experienced observer with a 95% intra-rater reliability manually scored the time that the marmoset spent: (1) exploring each object during the SOL task; (2) visually inspecting the snake stimulus during

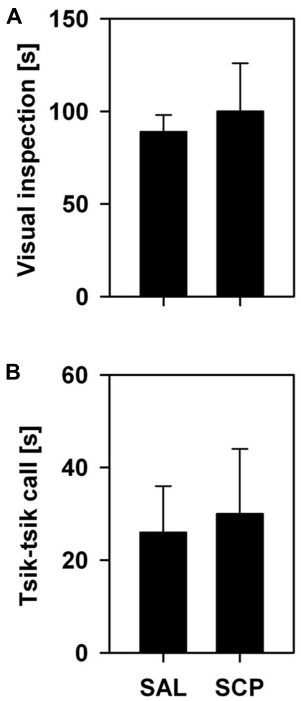

FIGURE 3 | Mean time (+SEM; in seconds) spent visually inspecting the snake model **(A)** and emitting *tsik–tsik* alarm vocalizations **(B)** by the saline (SAL; n = 4) and SCP (0.03 mg/kg SCP; n = 5) treated marmosets during the 10 min snake-exposure trial of the Fear-Motivated Behavior (FMB) procedure.

the FMB task; (3) emitting the alarm/mobbing-associated *tsik-tsik* calls during the FMB task; and (4) being vigilant during the FMB task. Exploration in the SOL task was defined as physical contact with one of the objects using the hands, feet, nose, mouth, or tongue, as well as all episodes of head cocks (side-to-side head movements), direct gazes (fast orientation of the eyes and head toward the object) and visual monitoring the object (continuous slow sweeping movements of the head). Visual inspection of the snake model included head cocks, direct gazes and visual monitoring of this object, whereas vigilance was defined as visual monitoring directed at the environment. Marmosets are highly visually oriented in their response to surrounding stimuli (Forster, 1995).

For the SOL task, all subjects were included in the analyses below as they met our pre-established criterion of exploring each object for at least 5 s during the sample trial. Recognition memory was operationally defined as a higher exploration of the displaced versus stationary object on the test trial (e.g., Dere et al., 2007), considering that captive marmosets preferably explore novel items in their environment (Forster, 1995). However, to account for individual variations in overall exploration levels, the following discrimination ratio was calculated based on Ennaceur et al. (1997): [time spent exploring the displaced object – time spent exploring the stationary object]/[time spent exploring both objects]. A ratio of $\approx$ 0.0 indicates that the two objects were explored almost equally (chance level), whereas a ratio $>$0.0 demonstrates that the displaced object was explored more than the stationary item. For the FMB procedure, we assessed the subjects' fear-induced place-avoidance response by comparing the time spent in the snake-paired section of the OF arena before and after the exposure trial (pre- x post-exposure trial).

Statistical Analyses

Data from males and females in each group were pooled together as the small sample size precluded any meaningful gender comparisons. For the SOL task, the time spent exploring the displaced versus stationary object on the test trial, as well as total exploration and distance traveled on the sample versus test trial, were analyzed using a mixed-design two-way analysis of variance (ANOVA), with 'treatment group' as the independent factor and 'object'/'trial' as the repeated measure variable. In addition, the discrimination ratios were compared to (zero value) chance-level performance via one-sample t-test. For the FMB task, an independent t-test was used for between-group comparisons regarding the visual inspection of the snake model and *tsik–tsik* vocalizations during the exposure trial. Dwell time in snake-paired quadrant, vigilance, distance traveled, and time spent in each corner section of the OF arena were analyzed via a mixed-design two-way ANOVA, with 'treatment group' as the independent factor and 'trial'/'section' as the repeated measure variable. Whenever significant effects were obtained in the ANOVA analyses, subsequent comparisons were performed using Tukey's test. Significance level for all tests was set at $p \leq 0.05$.

RESULTS

On the SOL test trial, the displaced object was explored for a significantly longer time than the stationary one, albeit only in the SAL-treated group (object effect: $F_{1,7} = 5.12$, $p = 0.05$; treatment effect: $F_{1,7} = 2.41$, $p = 0.17$; interaction: $F_{1,7} = 5.98$, $p = 0.04$; **Figure 2A**). The COC-treated animals explored both objects equivalently on the test trial. The SAL-treated marmosets explored the displaced object significantly above chance level on the test trial ($t_3 = 8.97$, $p = 0.003$), while the SCP group did not ($t_4 = -0.47$, $p = 0.67$; **Figure 2B**). This response was not significantly influenced by either a trial or treatment effect on the marmosets' overall exploration of the objects or by the level of locomotion, as both parameters remained constant between the sample and test trials (*object exploration* – trial effect: $F_{1,7} = 0.07$, $p = 0.80$; treatment effect: $F_{1,7} = 3.44$, $p = 0.11$; interaction: $F_{1,7} = 0.001$, $p = 0.97$; *distance traveled* – trial effect: $F_{1,7} = 0.11$, $p = 0.75$; treatment effect: $F_{1,7} = 0.86$, $p = 0.39$; interaction: $F_{1,7} = 0.30$, $p = 0.60$; **Figures 2C,D**).

During the initial baseline trial of the FMB task, held in the absence of the snake stimulus, all marmosets spent a comparable amount of time in the four corner quadrants of the OF arena (*SAL group* – section 1: 72 $\pm$ 18, section 2: 77 $\pm$ 19, section 3: 71 $\pm$ 13, section 4: 80 $\pm$ 9; *SCP group* – section 1: 69 $\pm$ 19, section 2: 78 $\pm$ 21, section 3: 74 $\pm$ 22, section 4: 78 $\pm$ 18; mean $\pm$ SEM in seconds; quadrant effect: $F_{3,21} = 0.09$, $p = 0.85$; treatment effect: $F_{1,7} = 0.01$, $p = 0.99$; interaction: $F_{3,21} = 0.02$, $p = 0.97$). On the conditioning trial, now in the presence of the snake

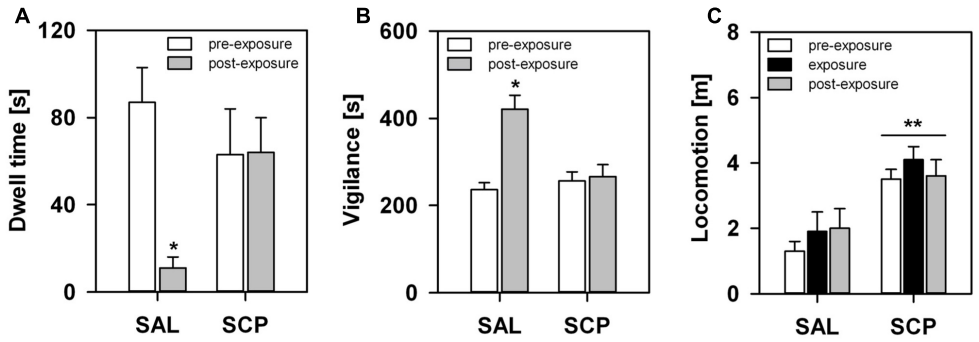

FIGURE 4 | Marmosets' behavioral response in the FMB procedure. An acute saline (SAL; n = 4) or SCP (0.03 mg/kg SCP; n = 5) administration was given subcutaneous immediately before the pre-exposure trial. All trials lasted 10 min and were held at 10 min intervals. Time spent in seconds **(A)** in the snake-paired quadrant of the open-field apparatus and **(B)** vigilant of the surroundings, before (pre-exposure trial) and after (post-exposure trial) being exposed to the snake model. **(C)** Total distance traveled in meters during each of the three trials of the procedure. All data are represented as mean + SEM. *$p < 0.05$ vs. the pre-exposure trial of the SAL group, **$p < 0.05$ SCP vs. SAL group.

model, the two groups also spent a similar amount of time visually inspecting the aversive stimulus ($t_7 = -0.36$, $p = 0.73$; **Figure 3A**) and emitting *tsik-tsik* alarm calls ($t_7 = -0.22$, $p = 0.84$; **Figure 3B**). However, after being confronted with the aversive stimulus, the SAL-treated marmosets spent significantly less time in the snake-paired quadrant of the OF apparatus compared to the levels seen prior to its exposure (baseline × test trial), whereas the SCP group spent a similar amount of time in this section on both trials (trial effect: $F_{1,7} = 5.80$, $p = 0.04$; treatment effect: $F_{1,7} = 0.75$, $p = 0.41$; interaction: $F_{1,7} = 5.92$, $p = 0.04$; **Figure 4A**). The SAL-treated marmosets were also found to be significantly more vigilant following the snake exposure, relative to the pre-confrontation levels of the baseline trial. Vigilance recorded in the SCP group remained unaltered between the baseline and test trials (trial effect: $F_{1,7} = 17.68$, $p = 0.004$; treatment effect: $F_{1,7} = 4.07$, $p = 0.08$; interaction: $F_{1,7} = 14.05$, $p = 0.007$; **Figure 4B**). Finally, the total distance traveled by the SCP-treated animals was significantly greater than that of the SAL group ($F_{1,7} = 21.94$, $p = 0.002$), however no between-trial effect ($F_{2,14} = 1.28$, $p = 0.30$) or trial-treatment interaction were observed ($F_{2,14} = 0.83$, $p = 0.43$; **Figure 4C**).

DISCUSSION

SCP-Induced Effects on the Spontaneous Spatial Recognition Memory

Our results showed that the nonselective muscarinic ACh receptor antagonist SCP impaired the marmosets' ability to detect changes in the spatial location of ethologically irrelevant stimuli in the environment. When assessed on the murine-based one-trial SOL recognition task (Ennaceur et al., 1997), SAL-treated animals explored the displaced object significantly longer than its identical stationary counterpart (i.e., exploration time and discrimination ratio). Captive callitrichids seem to readily respond to environmental change, particularly when spatial cues are involved (Gaudio and Snowdon, 2008) – an aspect possibly related to their use of highly seasonal habitats (Stevenson and Rylands, 1988). The exploratory preference for the displaced object in this group seems unlikely to be due to changes in object-related motivation or perception, or even overall activity, as total exploration and locomotor activity remained unaltered between the sample and test trials.

On the other hand, SCP-treated animals explored both objects equivalently during the test trial. To the best of our knowledge, NHPs have not yet been assessed in SOL tasks. The performance of rodents, however, is generally impaired following both systemic administrations (Murai et al., 2007; Pitsikas, 2007; Schäble et al., 2012) and local infusions of SCP into the perirhinal and medial prefrontal cortices (Barker and Warburton, 2009), as well as after selective immunotoxic lesions of central cholinergic systems (medial septum/vertical limb of the diagonal band; Easton et al., 2011). It is important to note as well that the SCP-treated marmosets explored both objects as much as the SAL group explored the displaced item. This was also the case when rodents were systemically administered the same antagonist (Schäble et al., 2012). As SCP was given 20 min prior to the sample trial, this treatment may have impaired the acquisition of relevant task-related information and thereby this group later perceived both objects as being novel rather than familiar. In rats, muscarinic blockade impaired the initial encoding phase of SOL, whilst sparing information retrieval (Barker and Warburton, 2009). This phase-dependent effect was also consistently shown in both rodents (e.g., Barker and Warburton, 2009) and NHPs (e.g., Aigner et al., 1991b) assessed in spontaneous and reinforced visual recognition tasks, respectively, as well as in healthy human volunteers (e.g., Atri et al., 2004). However, McTighe et al. (2010) found that direct damage to the perirhinal cortex led rodents to treat novel objects as familiar stimuli. Therefore, different factors may influence recognition memory processes, including the specific brain area involved, neurochemical mediator, task demands and animal model. Alternatively, the current SCP treatment may have impaired attentional and/or perceptual processes that are also required for the task (Voytko, 1996), yet this does not seem to be the case in our marmosets. Total object

exploration remained constant from the sample to the test trial, with no significant between-group differences being observed as well. Furthermore, we did not observe drug-induced changes in general locomotor activity. Although SCP has been shown to induce hyperactivity (Day et al., 1991), others reported a decrease (Besheer et al., 2001) or even a lack of effect (Schäble et al., 2012), leading to the suggestion that methodological aspects contribute significantly to the observed outcome (e.g., dose, behavioral task, administration route, gender; Klinkenberg and Blokland, 2010).

With the growing use of the several close variants of the spontaneous recognition memory task in rodents (Ameen-Ali et al., 2015), our results may have important prospective implications for the development of a preclinical cross-species procedure to assess specific memory functions. However, more comprehensive studies in marmosets are warranted, given that in rodents, for example, not all types of spontaneous recognition memories are affected by central cholinergic activity. Rats with selective immunotoxic lesions of specific basal forebrain cholinergic projections to the hippocampus were unable to recognize simple spatial representations (Easton et al., 2011), although other types of spatial memory tasks and more complex episodic-like memories remained intact with the lack of basal ACh input to the hippocampus or temporal/frontal cortex (Baxter et al., 1995; Easton et al., 2011). In marmosets, immunotoxic lesions of specific ACh neurons within the basal forebrain (Ridley et al., 1999) caused similar mnemonic deficits as those induced by ablation/excitotoxic damage to their respective target areas (Ridley et al., 1995; Barefoot et al., 2002) or even by SCP treatment (Harder et al., 1998), thus indicating that it may act more in terms of maintaining the proper functionality of their projection sites. If so, this rising signaling pathway probably affects long-term information encoding of different memory types, depending on its target structure (Harder et al., 1998). It is important to note that significant differences between rats and NHPs have been reported in terms of the cytochemical organization of basal forebrain cholinergic neurons, although the latter corresponded to that of humans (Geula et al., 1993). Therefore, it would be interesting to evaluate the effects of specific lesions on spontaneous recognition tasks in marmosets, as only visual discrimination and conditional learning tasks have been assessed. Pharmacological blockade of muscarinic receptors has consistently resulted in deficits in visual discrimination tasks of DNMS (reviewed in Robinson et al., 2011), similar to our current results in a spontaneous spatial recognition task. M1 receptor agonism was shown to enhance the cognitive performance and/or reverse SCP-induced deficits in these tasks (Rupniak et al., 1989; Carey et al., 1992; Harries et al., 1998; Lange et al., 2015).

SCP-Induced Effects on FMBs

We also demonstrated that the SCP-induced blockade of cholinergic neurotransmission disrupted the marmosets' ability to associate a predator-related stimulus with the specific spatial context in which it was encountered. On one hand, after a single brief encounter with the aversive stimulus, SAL-treated animals spent significantly less time in the specific snake-paired section of the OF arena, but more time being vigilant of their surroundings compared to pre-exposure levels. Concurrent changes in general activity were not observed. Snakes prey on marmosets (Teixeira et al., 2016), and as a result even inanimate related stimuli elicit a fear response in both feral and captive individuals (e.g., Barros et al., 2002). Le et al. (2013) have even argued that snakes exerted a prominent role in the development of primate neural structures, with minimal (Mineka et al., 1984) or no prior contact (Vitale et al., 1991) leading to persistently strong fearful reactions in NHPs. Exactly as we recorded in our subjects, during an encounter feral marmosets typically emit *tsik–tsik* alarm calls and visually inspect the snake; they never freeze (Ferrari and Lopes Ferrari, 1990; Teixeira et al., 2016). However, after the event, they act cautiously and avoid the interaction site for up to several days (Bartecki and Heymann, 1987). This indicates that: (1) our marmosets perceived the snake model as an unconditioned threat (e.g., Clara et al., 2008); (2) their post-encounter hypervigilance in the training context may be akin to the behavioral response of rodents during contextual fear-conditioning procedures using footshocks (*freezing*: reviewed in Maren et al., 2013) or predators (*risk assessment*: Ribeiro-Barbosa et al., 2005); and (3) subsequent avoidance of this specific location seems to be in line with the fear-induced conditioned-place-aversion (CPA) response seen in rodents (e.g., Zanoveli et al., 2007) and in NHPs under natural settings (Bartecki and Heymann, 1987; van Schaik and Mitrasetia, 1990; Isbell and Etting, 2017). Neurobiological studies on FMBs in NHPs is mostly focused on their unconditioned reaction to explicit aversive stimuli (e.g., predator, conspecifics), yet fear learning has been experimentally assessed using different paradigms, such as fear-potentiated startle (e.g., Antoniadis et al., 2009), cue-conditioning (Agustín-Pavón et al., 2012), observational conditioning (e.g., Mineka et al., 1984) and passive avoidance (e.g., Machado et al., 2009).

On the other hand, in the SCP-treated group, post-exposure vigilance and dwell time in the snake-paired section of the apparatus did not differ from the initial baseline levels of either group. This seems unlikely to be due to a drug-induced effect on their visual perception or behavioral response to the snake model. During their encounter with this stimulus we recorded similar levels of visual inspection and *tsik–tsik* alarm calls in both groups. The SCP group did, nonetheless, spend more time in motion than the SAL-treated animals. Although SCP may induce hyperactivity, as mentioned above, the difference was already present on the initial pre-exposure trial and as such may be a drug-unrelated feature inherent to that group.

The role of cholinergic signaling in fear learning of NHPs has yet to be fully addressed. Nonetheless, results from our present study seem to indicate that muscarinic antagonism may disrupt the encoding of conditioned fear responses for a spatial context in marmosets. In rodents, systemic and intra-hippocampal infusions of SCP selectively impaired the acquisition of a conditioned freezing response for the training context previously paired with an aversive footshock (recently reviewed in Wilson and Fadel, 2017). Selective antagonism of muscarinic M1 receptors (Soares et al., 2006) and pre-training electrolytic lesions of central cholinergic projections to the hippocampus yielded similar results (Maren and Fanselow, 1997). Cholinergic blockade also disrupts fear learning measured in

other behavioral tasks in rats (e.g., inhibitory avoidance; reviewed in Robinson et al., 2011). However, the role of muscarinic signaling on the retrieval of aversively motivated behavior is still unclear (reviewed in Robinson et al., 2011; Wilson and Fadel, 2017).

CONCLUSION

Our results indicate that the pharmacological blockade of cholinergic neurotransmission with SCP impaired the marmosets' ability to detect changes associated with the spatial location of ethologically irrelevant (SOL task) and relevant stimuli (FMB task). However, at present, we are only able to argue that cholinergic deficiency affects the way SOL recognition and aversive learning are processed in the short-term. Further studies are required to properly ascribe the role of ACh on the different phases of the information processing systems, their related brain circuits and the specific resultant effects. Similar investigations using longer retention intervals (>10 min), distinct objects/cues and gender comparisons will also contribute with important complementary information to our current understanding on normal and dysfunctional learning and memory processing in NHPs and potentially in humans. This novel approach, using a spontaneous (spatial) recognition task, may prove useful in terms of providing a means for a direct cross-species comparison between NHPs and rodents. Compared to other simians, the marmosets' small body size, rapid reproductive turnover, shorter life-span, high adaptability to captivity and lower husbandry costs (reviewed in Barros and Tomaz, 2002), while still retaining a high anatomical and neurochemical resemblance to their larger counterparts (Stephan et al., 1980; Geula et al., 1993), makes them a unique model for human neuropathologies. Marmosets may thus help reduce the translational gap between pre-clinical studies and memory-associated human pathologies.

AUTHOR CONTRIBUTIONS

JM, MB: conception and design; acquisition, analysis, and interpretation of data; drafting the article and revising it critically for important intellectual content. FdJ: conception and design; acquisition, analysis, and interpretation of data. RM: drafting the article and revising it critically for important intellectual content.

FUNDING

This study was supported by the Fundação de Apoio a Pesquisa do Distrito Federal (FAP-DF 193.001.026/2015). JM received a doctoral fellowship from the Brazilian Higher Education Authority (CAPES), FdJ a research scholarship from ProIC/UnB/CNPq, RS Maior a post-doctoral fellowship from CNPq (233647/2014-7) and MB a research fellowship from CNPq (304041/2015-7). These funding sources had no involvement in the study design, data collection, analysis or interpretation, writing the manuscript, or decision to submit it for publication.

ACKNOWLEDGMENTS

The authors would like to thank T. F. Roquete for assistance in data acquisition, A. P. Souto Maior for constructing the apparatus, and Drs. C. Dias and A. R. Souza, as well as G. V. da Silva and A. G. de Araújo for their dedicated care of the animals.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 10 August 2017
doi: 10.3389/fphar.2017.00530

Single Prazosin Infusion in Prelimbic Cortex Fosters Extinction of Amphetamine-Induced Conditioned Place Preference

*Emanuele C. Latagliata[1], Luisa Lo Iacono[1,2], Giulia Chiacchierini[2], Marco Sancandi[2], Alessandro Rava[3], Valeria Oliva[3] and Stefano Puglisi-Allegra[1,2]**

[1] Fondazione Santa Lucia IRCCS, Rome, Italy, [2] Dipartimento di Psicologia, Sapienza Università di Roma, Rome, Italy, [3] Dipartimento di Biologia e Biotecnologie "Charles Darwin", Sapienza Università di Roma, Rome, Italy

Exposure to drug-associated cues to induce extinction is a useful strategy to contrast cue-induced drug seeking. Norepinephrine (NE) transmission in medial prefrontal cortex has a role in the acquisition and extinction of conditioned place preference induced by amphetamine. We have reported recently that NE in prelimbic cortex delays extinction of amphetamine-induced conditioned place preference (CPP). A potential involvement of α1-adrenergic receptors in the extinction of appetitive conditioned response has been also suggested, although their role in prelimbic cortex has not been yet fully investigated. Here, we investigated the effects of the α1-adrenergic receptor antagonist prazosin infusion in the prelimbic cortex of C57BL/6J mice on expression and extinction of amphetamine-induced CPP. Acute prelimbic prazosin did not affect expression of amphetamine-induced CPP on the day of infusion, while in subsequent days it produced a clear-cut advance of extinction of preference for the compartment previously paired with amphetamine (Conditioned stimulus, CS). Moreover, prazosin-treated mice that had extinguished CS preference showed increased mRNA expression of brain-derived neurotrophic factor (*BDNF*) and post-synaptic density 95 (*PSD-95*) in the nucleus accumbens shell or core, respectively, thus suggesting that prelimbic α1-adrenergic receptor blockade triggers neural adaptations in subcortical areas that could contribute to the extinction of cue-induced drug-seeking behavior. These results show that the pharmacological blockade of α1-adrenergic receptors in prelimbic cortex by a single infusion is able to induce extinction of amphetamine-induced CPP long before control (vehicle) animals, an effect depending on contingent exposure to retrieval, since if infused far from or after reactivation it did not affect preference. Moreover, they suggest strongly that the behavioral effects depend on post-treatment neuroplasticity changes in corticolimbic network, triggered by a possible "priming" effect of prazosin, and point to a potential therapeutic power of the antagonist for maladaptive memories.

Keywords: α1-adrenergic receptors, extinction, prelimbic cortex, conditioned place preference, BDNF, PSD-95, nucleus accumbens

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Miroljub Popovic, Universidad de Murcia, Spain
Cristiano Chiamulera, University of Verona, Italy

***Correspondence:**
Stefano Puglisi-Allegra
stefano.puglisi-allegra@uniroma1.it

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *30 June 2017*
Accepted: *28 July 2017*
Published: *10 August 2017*

Citation:
Latagliata EC, Lo Iacono L, Chiacchierini G, Sancandi M, Rava A, Oliva V and Puglisi-Allegra S (2017) Single Prazosin Infusion in Prelimbic Cortex Fosters Extinction of Amphetamine-Induced Conditioned Place Preference. Front. Pharmacol. 8:530. doi: 10.3389/fphar.2017.00530

INTRODUCTION

Persistent memories about biologically relevant stimuli are essentials for organism and species survival. However, in some pathological conditions, highly salient memories are experienced intrusively leading the individual to maladaptive behavior (McGaugh, 2006; Sun et al., 2011; Puglisi-Allegra and Ventura, 2012). A typical example is drug addiction, a pathological condition, in which environmental stimuli or discrete cue paired with drug effects acquire the ability to induce intense drug desire that leads to drug-seeking and drug-taking (Stewart et al., 1984; Robinson and Berridge, 1993; Childress et al., 1999; Everitt et al., 2001; Shalev et al., 2002). A method to reduce this kind of responses is the extinction learning, a protocol in which repeated cues or context re-exposure in absence of the predicted event results in a decrease in magnitude and frequency of conditioned response (CR) (Myers and Davis, 2004; Myers and Carlezon, 2012). This protocol, known as exposure therapy, has been applied successfully in fear and anxiety disorders treatment. However, its application on drug addicts showed only limited success (Conklin and Tiffany, 2002). Therefore, a better understanding of neurobiological mechanisms of extinction could be important to improve the effectiveness of extinction-like protocols, and to envisage neural targets to develop pharmacological therapy.

Modulation of the conditioned stimulus (CS) motivational properties favors disengagement from drug-related cues. Thus, therapy strategies aimed at reducing the motivational properties of drug cues have been considered highly promising for successful treatment of craving and relapse in addicts (Taylor et al., 2009). In this framework, norepinephrine (NE) transmission in prefrontal cortex acquires a pivotal role to favor disengagement from drug-related cues.

Indeed, NE transmission in mPFC modulates central and behavioral responses induced by relevant biological stimuli or by neutral stimuli associated with them (Darracq et al., 1998; Feenstra et al., 2001; Mingote et al., 2004; Ventura et al., 2005, 2007, 2008; Pascucci et al., 2007; Puglisi-Allegra and Ventura, 2012).

Drugs of abuse, natural reward and aversive stimuli promotes NE increase in mPFC (Florin et al., 1994; Feenstra et al., 2001; Mingote et al., 2004; Ventura et al., 2005, 2007; Pascucci et al., 2007), leading to dopamine (DA) release in the NAc that is critical for the attribution of motivational salience to highly salient stimuli (Darracq et al., 1998; Ventura et al., 2003, 2005, 2007; Puglisi-Allegra and Ventura, 2012). Furthermore, highly salient unconditioned stimulus (US) and conditioned stimulus (CS) paired with them, increase NE levels in mPFC proportionally to the salience of the US (Feenstra et al., 2001; Mingote et al., 2004; Ventura et al., 2008). This indicates that prefrontal NE transmission modulates motivational properties of conditioned cues during exposure to them, strengthening them and contributing to the maintenance of the CR. Consistent with this view, we have recently reported that selective NE depletion in pre-limbic cortex (PL), after acquisition of amphetamine CPP, facilitates extinction of drug-associated memory (Latagliata et al., 2016).

Alpha1-adrenergic receptors (α1-ARs) in mPFC have been related to the modulation of motivational properties of salient experiences. Indeed, α1-ARs that are preferentially engaged during conditions of sustained prefrontal NE release (Ramos and Arnsten, 2007) like highly salient experiences (Mingote et al., 2004; Ventura et al., 2008), modulate both motivated behavior and dopaminergic response in NAc induced by salient stimuli (Blanc et al., 1994; Darracq et al., 1998; NicNiocaill and Gratton, 2007; Schmidt et al., 2017).

Thus, in the present work we investigated if α1-ARs in PL cortex are involved in extinction of amphetamine-induced CPP in mice.

Note that the selective α1-ARs antagonist prazosin is used in clinical set for treatment of alcohol abuse and for post-traumatic stress disorder (Raskind et al., 2003; Rasmussen et al., 2009). Moreover, one of the pioneering studies by the Collège de France team (Blanc et al., 1994) showed that infusion of prazosin in the mpFC was able to control DA functioning in the NAc along more than 1 day after treatment. This report encouraged us to use prazosin in our experimental conditions in order to assess the effects of a single treatment in animal exposed to extinction training to possibly benefit from long-lasting pharmacological effects of the compound.

Thus, in the present work we investigated the effects of prazosin infusion in the PL of C57BL/6J mice on expression and extinction of amphetamine-induced CPP, hypothesizing that a single intra-PL prazosin infusion, before CPP trial (reactivation), could reduce the motivational properties of the amphetamine paired CS, weakening the persistence of amphetamine-induced CPP.

In a first set of experiments, we observed that intra-PL prazosin did not affect expression of amphetamine-induced CPP the day of infusion, while in subsequent days, when animals were drug free, it produced an early extinction in comparison with vehicle infused group. This suggested that PL α1-ARs blockade could have induced neuroplastic adaptations in mesocorticolimbic areas of prefrontal-accumbal network known to modulate incentive salience and extinction.

To assess this hypothesis, in a second set of experiments, we assessed the transcriptional modulation of BDNF and PSD-95 genes as markers of neuroplasticity and synaptic maturation, respectively, in PL and infralimbic cortex (IL), nucleus accumbens (NAc) shell and core, of prazosin treated mice having extinguished preference for amphetamine CS.

MATERIALS AND METHODS

Animals

Male C57BL/6JIco (Charles River, Como, Italy) were purchased at 6–7 weeks of age and housed four per cage on a 12-h light–dark cycle (lights on between 07.00 a.m. and 07.00 p.m.) for 3 weeks. Two days before experiments, animals were individually

Abbreviations: α1-ARs, alpha1-adrenergic receptors; Amph, amphetamine sulfate; BDNF, brain-derived neurotrophic factor; CPP, conditioned place preference; IL, infralimbic mpFC; mpFC, medial prefrontal cortex; NAc, nucleus accumbens; PL, prelimbic mpFC; PSD-95, post-synaptic density 95.

housed. Each experimental group consisted of 7–10 animals. All experiments were carried out in accordance with Italian national law (DL 116/92 and DL 26/2014) on the use of animals and with the European Communities Council Directives (86/609/EEC and 2010/63/UE), and approved by the ethics committee of the Italian Ministry of Health (license/approval ID #: 10/2011-B and 42/2015-PR).

Drugs

D-Amphetamine sulfate (Amph) and Prazosin hydrochloride (prazosin), were purchased from Sigma (Sigma Aldrich, Milan, Italy). Fluorescently labeled prazosin, BODYPY FL, was purchased by Thermo Fisher Scientific, Italy. Amph (2.5 mg/Kg), was dissolved in saline (0.9% NaCl) and injected intraperitoneally (i.p.) in a volume of 10 ml/kg. Zoletil 100, Virbac, Milan, Italy (tiletamine HCl 50 mg/ml + zolazepam HCl 50 mg/ml) and Rompun 20, Bayer S.p.A Milano, Italy (xylazine 20 mg/ml), purchased commercially, were used as anesthetics. Prazosin and BODYPY FL (1mg/ml) were dissolved in artificial CSF (in mM: NaCl 140.0; KCl 4.0; $CaCl_2$ 1.2; $MgCl_2$ 1.0). Artificial CSF was used as Vehicle. The doses of Amph and prazosin were selected on the bases of previous studies (Darracq et al., 1998; Do-Monte et al., 2013; Latagliata et al., 2016) and preliminary experiments.

Apparatus

A CPP apparatus (Cabib et al., 1996, 2000) was used for behavioral experiments. The apparatus comprised two gray Plexiglas chambers (15 cm × 15 cm × 20 cm) and a central alley (15 cm × 5 cm × 20 cm). Two sliding doors (4 cm × 4 cm) connected the alley to the chambers. In each chamber, two triangular parallelepipeds (5 cm × 5 cm × 20 cm) made of black Plexiglas and arranged in different patterns (always covering the same surface of the chamber) were used as conditioned stimuli. Behavioral data were registered and analyzed by a fully automated video tracking system ("EthoVision", Noldus Information Technology, Wageningen, The Netherlands). The acquired digital signal was processed by the software, to extract the "time spent" [in second (s)] in the three chambers of the apparatus.

Experimental Procedures

Experimental procedures are summarized in **Figure 1**.

Conditioned Place Preference

The training procedure was described previously (Cabib et al., 1996). Briefly, on day 1 (pre-test), mice were free to explore the entire apparatus for 20 min. On the following 8 days (conditioning phase), mice were injected and confined daily for 40 min alternatively in one of the two chambers. One of the patterns was consistently paired with a vehicle injection and the other one with Amph injection. In order to balance the pairings for half of the animals in each experimental group Amph (or vehicle) was paired with one of the patterns and half of them with the other one. Testing was carried out on day 10 in drug-free state and lasted 20 min like the pre-test. Note that during pre-test, mice randomly assigned to vehicle ($n = 10$) or prazosin ($n = 10$) groups did not show preference (mean time spent ± SEM), for the lateral chambers, thus showing that the apparatus was unbiased in terms of preferences in untreated mice.

Surgery, Re-test and Infusions

The days following CPP test (days 11 and 12), animals were subjected to surgical procedures. Mice, anesthetized with Zoletil 100 and Rompun 20, were mounted in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, United States) equipped with a mouse adapter. An incision was performed along the midline of the skull, then two holes were pierced in correspondence of the PL cortex, coordinates: AP +2.8; ML ± 0.4 DV −0.4 from the bregma, according to the atlas of Paxinos and Franklin (2001). Two steel cannulas were implanted (length: 7 mm; outer diameter: 0.7 mm, internal diameter 0.35 mm), secured with dental cement with the addition of epoxy glue.

One week after CPP test, the animals were subjected to a further test (re-test) to check that surgeries did not impair place preference. The day following CPP re-rest, bilateral injections of prazosin or vehicle (0.6 μg/0.6 μl side) were performed into PL through a stainless steel cannula (length 8.1 mm, 0.15 mm outer diameter, UNIMED, Swiss), connected to a 10 μl Hamilton syringe by a polyethylene tube and driven by a CMA/100 pump (flow rate 0.6 μl/min). After the end of the infusion the cannula was left in place for additional 30 s. Vehicle or prazosin pre-trial group received the infusion 20 min before the CPP trial. Post trial groups (vehicle or prazosin) received injection in PL 5 min after CPP trial. Non-contingent groups received PL infusion of vehicle or prazosin 5–6 h before the CPP trial.

Placement Assessment

To assess placement, drug dispersion, and tissue damage in the PL a solution of a CSF containing the fluorescently labeled BODYPY FL prazosin was infused as described before. **Figure 2** shows a representative image of the preparation used to determine location of the cannula in the PL after infusion with CSF (**Figure 2A**) or fluorescently labeled prazosin (**Figure 2B**).

Placements in PL were judged by methylene blue staining. Brains were post-fixed in 4% paraformaldehyde, cut in serial coronal slices according to Paxinos and Franklin (2001) and processed for methylene blue staining. In order to reconstruct the correct cannula placements 50-μm thick sections were examined under a microscope to establish the location of the cannula. In **Figure 2** is represented the location of cannulas in the two hemispheres. Data from animals not showing the proper placement ($n = 9$ for all experiments) were discarded from the final statistical analysis.

Extinction

The extinction procedure began the day after the re-test. To investigate potential time-dependent differences in the extinction, animals were exposed daily to CPP test (20 min) (Orsini et al., 2008) (non-confined extinction). The extinction of the CR was considered acquired after two consecutive days showing non-significant preference for the drug-paired chamber (Fricks-Gleason and Marshall, 2008; Latagliata et al., 2016).

Day							
1	2-9	10	11-12	13-16	17	18	19-33
Pre-Test	Conditioning Phase	Test	Surgery	Post Surgery Recovery	Re-Test	Infusion	Non-Confined Extinction Procedure (Daily Test Session)
						Pre-Trial 20 min before CPP	
						Post-Trial 5 min after CPP	
						Non-Contingent 5-6 hours before CPP	

FIGURE 1 | Schematic time-line of the experimental procedures.

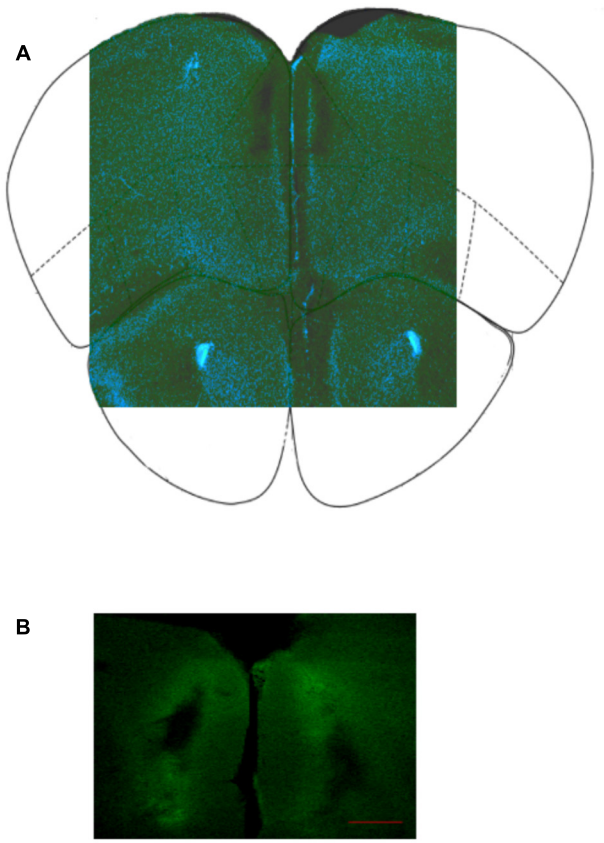

FIGURE 2 | (A) Representative image of infusion site in PL cortex; **(B)** Location of bilateral cannula insertion to infuse fluorescently labeled prazosin (BODYPY FL) in the prelimbic cortex, PL (see Materials and Methods). The image is representative coronal photomicrographs of PL. The fluorescent trace was used to evaluate the selectivity of the diffusion gradient. The clearest part indicates the maximum spread of prazosin in the PL around the cannula track. Scale bar = 200 μm.

Quantitative Real Time RT-PCR and Gene Expression Analysis

After extinction of prazosin treated mice, the animals of the four experimental groups were sacrificed, brains were removed and stored in liquid nitrogen. Then, after brains were fixed vertically on the freeze plate of a freezing microtome, punches of both hemispheres were obtained from the brain slices (coronal sections) no thicker than 300 μm. Stainless steel tubes of 1.0 mm of inside diameter for PL and 0.5 mm for IL, NAc Core and NAc Shell were used. The coordinates were measured according to the Paxinos and Franklin (2001) atlas (coronal sections as mm from bregma), as follows: PL two slices from 2.80 to 2.22; IL two slices from 2.10 to 1.54; NAc Core and Shell three slices from 1.88 to 0.98. The punches were stored in liquid nitrogen until the day of RNA extraction.

RNA was isolated from brain punches using Total RNA purification Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer protocol. RNA quantity was determined by absorbance at 260 nm using a NanoDrop UV-VIS spectrophotometer. Complementary DNA was obtained using the High Capacity Reverse Transcription Kit (Applied Biosystems, Branchburg, NJ, United States). cDNA templates (8 ng) were amplified with quantitative PCR using the Taqman technology in the 7900HT thermal cycler apparatus equipped with the SDS software version 2.3 (Applied Biosystems) for data collection. Taqman primer sets (Applied Biosystems) were used to amplify mouse total *BDNF* (Mm04230607_s1; amplifying the coding region for mature BDNF) and Disks large homolog 4 (Dlg4 Mm00492193_m1), gene encoding for *PSD-95*. Ct values were normalized to measures of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH Mm99999915_g1) mRNA. All data were run in triplicate and analyzed using the ΔΔC(t) method (Schmittgen and Livak, 2008). Results are expressed as fold changes relative to the correspondent vehicle-treated group.

Statistics

Time spent (s) in each of the three chambers was used as a dependent measure. Data were analyzed by repeated-measures ANOVA with one between factor (treatment, two levels: vehicle, prazosin) and one within factor (choice, three levels: center, paired, and unpaired). *Post hoc* comparisons were assessed by Duncan's multiple-range test whenever significant main effects were attained. A significant CPP was indicated by a significant difference between time spent in paired versus unpaired chamber.

BDNF and *PSD-95* mRNA levels of expression were compared between prazosin and vehicle group by paired *t*-test with significant values attributed when $p < 0.05$.

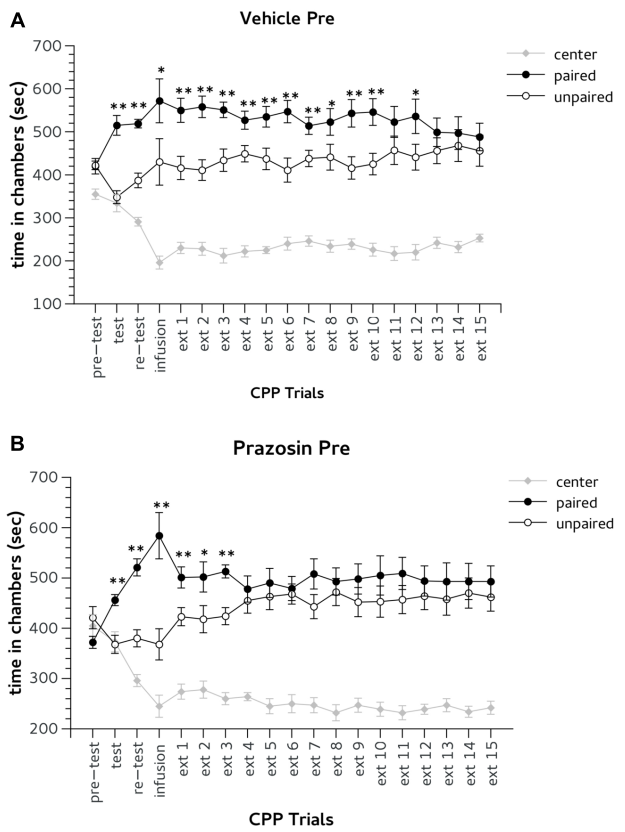

FIGURE 3 | Effects of pre-trial infusion of prazosin in prelimbic (PL) prefrontal cortex on expression and extinction of an acquired conditioned place preference (CPP) induced by systemic injection of 2.5 mg/Kg of amphetamine. In the *x*-axis are days. In the *y*-axis is time spent in center, paired, and unpaired chamber during pre-test, test, re-test, and non-confined extinction trials in animals assigned to vehicle **(A)** and prazosin **(B)**. All data are expressed as mean (second ± SE) time spent in center, paired, and unpaired chambers. $^{*}p < 0.05$, $^{**}p < 0.01$ in time spent in paired in comparison with unpaired chamber in vehicle and prazosin infused mice.

RESULTS

Prelimbic Prazosin Infusion Fosters Extinction of Conditioned Place Preference Induced by Amphetamine

We first investigated the effects of PL α1-ARs antagonist infusion on expression and extinction of Amph induced CPP. Two-way ANOVA showed non-significant (ns) effect for treatment × choice interaction: $F(2,36) = 2.668$, ns and for choice: $F(2,36) = 1.932$, ns.

Following conditioning (test) and in the trial after surgery (re-test), all mice expressed a preference for the previously Amph-paired chamber (**Figures 3A,B**). Two-way ANOVA revealed a significant effect for factor choice: [test, $F(2,36) = 20.865$, $p < 0.01$; re-test, $F(2,36) = 80.789$, $p < 0.01$]. Duncan's *post hoc* analysis showed that both vehicle and prazosin animals spent more time in the Amph-paired chamber during test and re-test ($p < 0.01$) (**Figures 3A,B**).

Bilateral infusion of both prazosin and vehicle performed 20 min before the third CPP trial (infusion, **Figure 1**) did not affect the expression of Amph CPP (**Figures 3A,B**). Two-way ANOVA showed a significant effect for choice: [infusion, $F(2,36) = 26.705$; $p < 0.01$] and *post hoc* analysis confirmed that both groups spent more time in Amph-paired chamber during this trial (prazosin $p < 0.01$; vehicle $p < 0.05$) (**Figures 3A,B**). On extinction trial 5, mice infused with prazosin reached extinction criterion of two consecutive days showing non-significant preference for the drug-paired chamber (**Figure 3B**), whereas vehicle treated reached extinction on trial 14 (**Figure 3A**). On extinction trials 4 and 5, respectively, statistical analyses revealed a significant effect of choice [$F(2,36) = 62.186$; $p < 0.01$] and [$F(2,36) = 54.946$; $p < 0.01$]. *Post hoc* test showed that on both days only vehicle spent more time in the Amph- than in the saline-paired chamber ($p < 0.01$) (**Figure 3A**).

Recently, it has been shown that PL prazosin infusion can impair fear memory re-consolidation leading to attenuation of fear responses (Do-Monte et al., 2013). To evaluate a potential re-consolidation effect in our results we performed a second experiment in which prazosin was administrated in PL cortex 5 min after CPP trial (see **Figure 1**).

As in the previous experiment, during pre-test mice randomly assigned to vehicle post trial ($n = 7$) or prazosin post trial ($n = 7$) groups did not show preference for the lateral chambers. Two-way ANOVA revealed non-significant effect for interaction group × choice: $F(2,24) = 0.10$, ns and for factor choice: $F(2,24) = 12.994$, ns.

In both CPP test and re-test all mice showed the preference for Amph paired chamber. Two-way ANOVA showed a significant effect for factor choice: [test, $F(2,24) = 55.705$; $p < 0.01$; re-test, $F(2,24) = 54.955$; $p < 0.01$]. The day after post-trial infusion, extinction (ext) 1, both groups vehicle and prazosin showed CPP for Amph paired chamber. Two-way ANOVA showed a significant effect for factor choice during ext 1: $F(2,24) = 47.082$; $p < 0.01$, but non-significant interaction treatment × choice: $F(2,24) = 0.06$, ns (**Figures 4A,B**). Prazosin post-trial group reached extinction criterion on the fourteenth trial. Statistical analyses revealed significant effect of choice for both ext 13 [$F(2,24) = 41.957$; $p < 0.01$] and ext 14 [$F(2,24) = 29.457$; $p < 0.01$]. However, Duncan's test showed that in both days prazosin treated mice did not show difference in time spent in Amph- in comparison with saline-paired chamber (**Figure 4B**). Likewise, vehicle post-trial group reached the extinction criterion on the 15th trial. Two-way ANOVA revealed significant effect of the factor choice in both ext 14 [$F(2,24) = 29.457$; $p < 0.01$] and ext 15 [$F(2,24) = 23,642$; $p < 0.01$]. Duncan's test showed that in both days vehicle post-trial mice have spent an equal amount of time in the chambers paired with Amph and saline (**Figure 4A**).

Finally, we verified whether PL prazosin infusion produced a facilitator effect on the extinction of Amph-induced CPP independently by the exposure to the CPP trial context. In this experiment prazosin or vehicle were administrated 5–6 h before the CPP trial (non-contingent groups). During pre-test mice randomly assigned to non-contingent vehicle

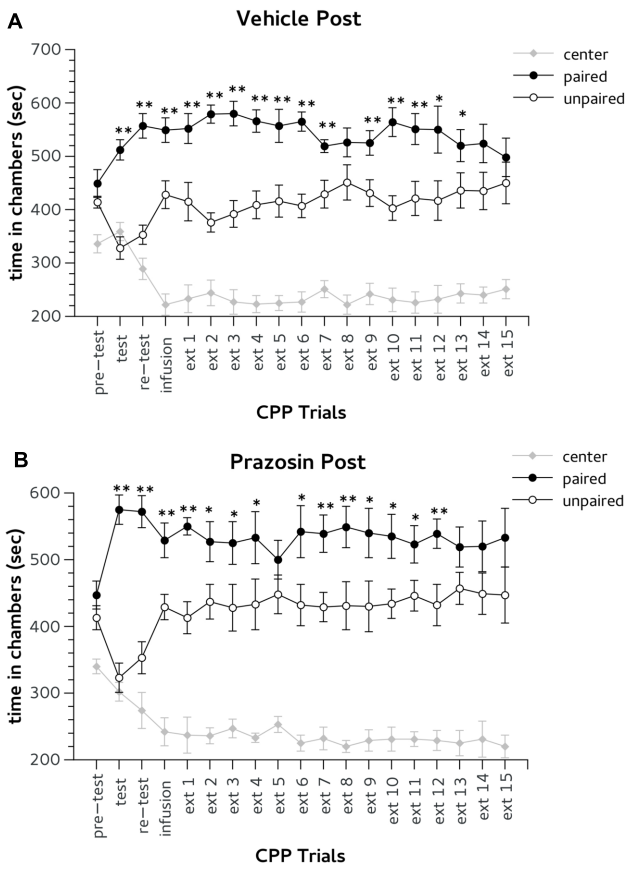

FIGURE 4 | Effects of post-trial infusion of prazosin in prelimbic (PL) prefrontal cortex on expression and extinction of an acquired conditioned place preference (CPP) induced by systemic injection of 2.5 mg/Kg of amphetamine. In the *x*-axis are days. In the *y*-axis is time spent in center, paired, and unpaired chamber during pre-test, test, re-test, and non-confined extinction trials in animals assigned to vehicle **(A)** and prazosin **(B)**. All data are expressed as mean (second ± SE) time spent in center, paired, and unpaired chambers. $*p < 0.05$, $**p < 0.01$ in time spent in paired in comparison with unpaired chamber in vehicle and prazosin infused mice.

($n = 8$) or prazosin ($n = 10$) groups did not show preference for the lateral chambers (**Figures 5A,B**). Two-way ANOVA revealed non-significant effect for treatment × choice interaction: $F(2,32) = 0.423$, ns. The factor choice was significant: $F(2,32) = 11.332$, $p < 0.01$. Duncan's *post hoc* test confirmed that both groups spent a greater amount of time in two lateral chambers in comparison with the central alley.

During CPP test and re-test all mice showed the preference for Amph paired chamber. Two-way ANOVA showed a significant effect for factor choice: [test, $F(2,32) = 40.386$, $p < 0.01$; re-test, $F(2,32) = 42.204$, $p < 0.01$]. In the CPP trial after non-contingent infusion both groups showed a preference for the Amph-paired chamber (**Figures 5A,B**). ANOVA revealed no significant effect for treatment × choice interaction: $F = (2,32) = 0.850$, ns; but a significant effect for the factor choice: $F = (2,32) = 41,252$, $p < 0.01$. Non-contingently prazosin infused mice reached the extinction criterion on trial 8. ANOVA revealed a significant effect for the factor choice in both ext 7 [$F = (2,32) = 47,797$, $p < 0.01$] and 8 [$F = (2,32) = 50,338$, $p < 0.01$]. However, Duncan's test showed that in both days only non-contingent vehicle mice spent more time in the chamber previously paired with ($p < 0.01$) (**Figure 5A**). Note, that non-contingent prazosin mice showed a spontaneous recovery for Amph CPP during extinction trial 13 (**Figure 5B**). Non-contingent vehicle group reached the criterion of two consecutive daily lack of preference on the 15th trial. Two-way ANOVA showed a significant effect of the factor choice in both ext (ext 14 [$F(2,32) = 34.749$, $p < 0.01$] and 15 [$F(2,32) = 25,623$, $p < 0.01$]. Duncan's test showed that in both days vehicle non-contingent mice spent an equal amount of time in the chambers paired with Amph and vehicle (**Figure 5A**).

Prelimbic Prazosin Infusion, Immediately before the Exposure to the CPP Trial, Increases BDNF and PSD-95 mRNA Expression in the Nucleus Accumbens

Given the delayed effect of the intra-PL prazosin infusion on the extinction of Amph-induced CPP, we hypothesized that the acute blockade of PL α1-ARs contingent with the exposure to the CPP trial context may induce neuroplastic adaptations in the prefrontal-accumbal network, that underlie the facilitation of extinction. We thus analyzed by quantitative real-time PCR the transcription levels of the neurotrophin *BDNF* and of the synaptic scaffold *PSD-95* as markers of neuroplasticity and synaptic maturation, respectively. Gene expression was measured in punches of IL and PL cortices, and NAc core and shell subregions, of pre-trial prazosin- and vehicle-treated mice. To ensure that both vehicle and prazosin group were equally exposed to extinction trials, mice were sacrificed for mRNA assessment once the preference for Amph CS was extinguished only in prazosin-treated mice, but not necessarily in the vehicle.

For *BDNF* analysis, paired *t*-test revealed a significant increase of the *BDNF* transcript in NAc core punches of pre-trial PL prazosin-infused mice compared to vehicle [$t(4) = 3.877$, $p < 0.05$]. Non-significant effect was observed in NAc shell [$t(4) = 0.888$, ns], PL [$t(3) = -0.643$, ns] or IL [$t(4) = -0,697$, ns] (**Figure 6**). Similarly, the *PSD-95* mRNA expression level increased significantly in NAc shell punches of pre-trial PL prazosin-infused mice compared to vehicle [$t(4) = 2.801$, $p < 0.05$], while no effect of the drug was observed in NAc core [$t(3) = 2.315$, ns], PL [$t(3) = -0.197$, ns)] or IL [$t(4) = 0.049$, ns] (**Figure 7**). Note that a trend toward an increase was evident in NAc core.

To test the possibility that the PL prazosin infusion would induce the observed transcriptional modulation independently of the contingent exposure to the CPP trial context, we measured mRNA levels of *BDNF* and *PSD-95* in non-contingently infused prazosin or vehicle mice again when the compartment-preference was extinguished. For *BDNF* analysis, paired *t*-test revealed a significant decrease in the *BDNF* transcript in NAc shell punches of non-contingently PL-infused prazosin mice compared to vehicle [$t(6) = 2.518$, $p < 0.05$). No differences were observed

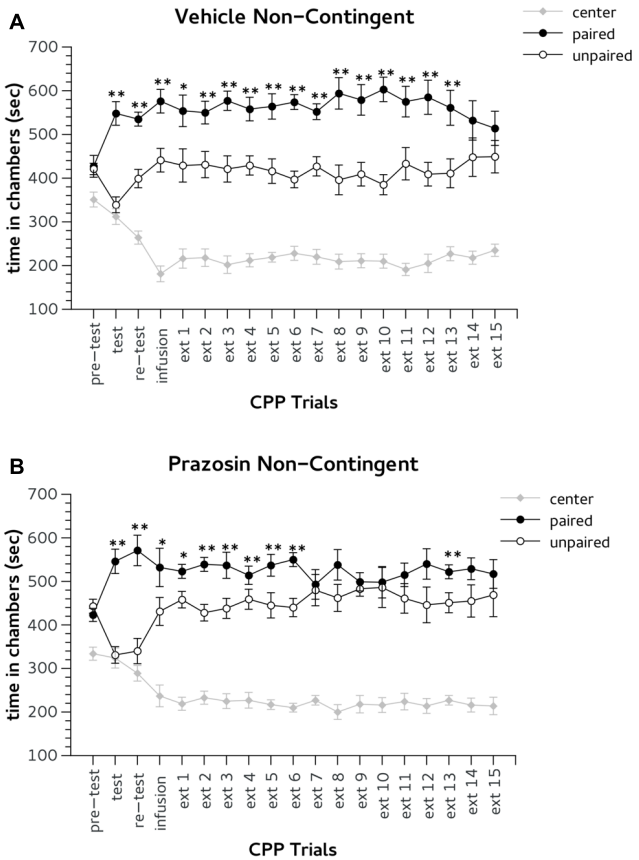

FIGURE 5 | Effects of non-contingent infusion of prazosin in prelimbic (PL) prefrontal cortex on expression and extinction of an acquired conditioned place preference (CPP) induced by systemic injection of 2.5 mg/Kg of amphetamine. In the *x*-axis are days. In the *y*-axis is time spent in center, paired, and unpaired chamber during pre-test, test, re-test, and non-confined extinction trials in animals assigned to vehicle **(A)** and prazosin **(B)**. All data are expressed as mean (second ± SE) time spent in center, paired, and unpaired chambers. $^{*}p < 0.05$, $^{**}p < 0.01$ in time spent in paired in comparison with unpaired chamber in vehicle and prazosin infused mice.

in NAc core [$t(5) = 1.366$, ns], PL [$t(2) = 0.991$, ns], and IL [$t(2) = -0.087$, ns] (**Figure 8**). Non-significant effect of the drug was revealed for the *PSD-95*, in either structure: NAc shell [$t(6) = 0.661$, ns], NAc core [$t(5) = -1.013$, ns], PL [$t(2) = 1.034$, ns], and IL [$t(1) = -0.332$, ns] (**Figure 9**).

DISCUSSION

This study was aimed at evaluating the role of PL α1-ARs in the maintenance of CPP to Amph. To this aim we assessed the effects of a single pre-reactivation infusion of prazosin in the PL of C57BL/6J mice on the expression of a previously acquired Amph-induced CPP the day of infusion and the following days, when mice were tested drug free. Intra-PL prazosin did not affect expression of Amph-induced CPP the day of infusion, while in subsequent days it produced a clear-cut advance of extinction in comparison with vehicle treated animals. Indeed, from day 5 onward prazosin-treated mice, exposed to the apparatus, showed no preference for the drug-paired chamber differently from vehicle group that extinguished preference for the paired chamber on day 14. Note that this effect of intra-PL prazosin priming is dependent on its infusion before reactivation, since if infused far before or after reactivation it did not affect significantly preference compared with vehicle groups.

A beta-adrenergic receptor antagonist, infused in this area before CPP test, has been reported to block the expression of cocaine-seeking the day of infusion and on the following day when animals are drug-free (Otis et al., 2013). However, in the present study a possible retrieval effect or general memory impairment can be ruled out. Indeed, before the onset of extinction procedure animals assigned to both vehicle and prazosin treatment were able to retrieve drug-associated memory in CPP expressing Amph preference for the paired chamber.

We observed here that no extinction occurred the day of infusion when preference for paired chamber was similar to that shown by vehicle group while preference in treated mice was no more evident after subsequent daily testing sessions. Therefore, a mechanism that could account for not immediately extinction occurring following infusion should have been searched in possible neuroplasticity events triggered by intra-PL prazosin. These could have tagged CS during CPP retrieval, possibly devaluating it or blunting its association with US to produce a process that led to "extinction" of preference later on.

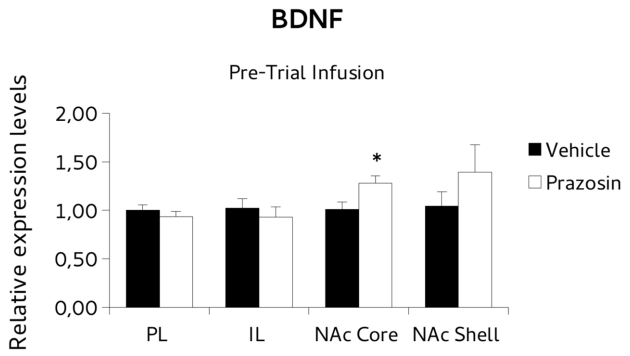

FIGURE 6 | Brain-derived neurotrophic factor (BDNF) mRNA expression in punches of infralimbic (IL) and prelimbic (PL) cortices, and Nucleus Accumbens (NAc) core and shell subregions, measured after the extinction of amphetamine-induced conditioned place preference (CPP), in mice that received pre-trial infusion of prazosin or vehicle in PL. The BDNF mRNA expression level (relative expression levels = fold changes over vehicle, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene) was significantly increased in the NAc core of Prazosin versus Vehicle-infused mice. $*p < 0.05$.

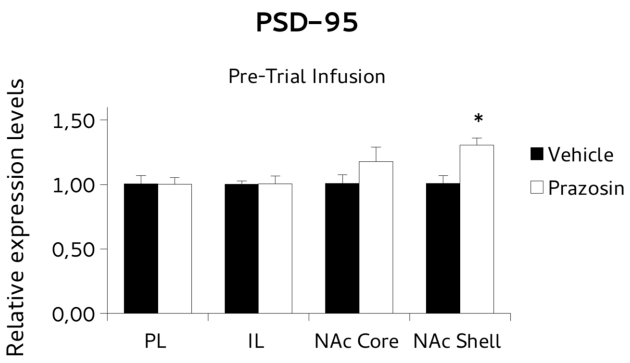

FIGURE 7 | Postsynaptic density protein 95 (PSD-95) mRNA expression in punches of IL and PL cortices, and NAc core and shell subregions, measured after the extinction of amphetamine-induced CPP, in mice that received pre-trial infusion of prazosin or vehicle in PL. The PSD-95 mRNA expression level (relative expression levels = fold changes over vehicle, normalized to GAPDH gene) was significantly increased in the NAc shell of Prazosin versus Vehicle-infused mice. $*p < 0.05$.

Such neuroplasticity events could have involved not only the brain areas where the antagonist was infused but also other areas that are known to be connected with it and playing a functional role in expression, reconsolidation or extinction of association between CS and drugs.

Lasting efficacy of prazosin in the short period could not be ruled out (Blanc et al., 1994); however, if the compound were effective in testing sessions following intra PL infusion, behavioral effects (extinction) should have been evident also in mice treated non-contingently with exposure to the apparatus. Moreover, post-retrieval treatment did not affect place preference significantly, thus ruling out a possible interference with consolidation of reconsolidation (Dudai and Eisenberg, 2004) produced by intra-PL prazosin prior reactivation due to long-lasting pharmacological action.

To evaluate neuroplastic adaptations we chose to analyze the transcriptional modulation of *BDNF* and *PSD-95 genes*. BDNF is a neurotrophin that is critically involved in the activity-dependent regulation of synaptic structure and function and thus it is considered a reliable marker of neuronal plasticity. BDNF is released upon neuronal activation and exerts its effect on synaptic strength, modulation of dendritic growth, changes in spine density and morphology, by stimulation of protein synthesis and transcription activity, which account for its delayed effect (Carvalho et al., 2008). Interestingly BDNF-induced neuroplasticity has been shown to induce extinction (Rosas-Vidal et al., 2014).

PSD-95 is the most abundant scaffolding protein at mature glutamate synapses and is essential for synaptic maturation and plasticity (El-Husseini et al., 2000). BDNF signaling is known to promote PSD-95 translation and trigger transport of PSD-95 to the synapse where it contributes to the structural and functional maturation of synapses (Yoshii et al., 2011).

In our experiment we assessed transcriptional expression of *BDNF* and *PSD-95* in mice that had extinguished CPP. Specifically, we evaluated mRNA levels in PL and IL cortices and NAc subdivision shell and core (areas of a prefrontal-accumbal network modulating incentive salience and extinction).

The results showed that prazosin-treated mice having extinguished preference showed an increase in *BDNF* mRNA expression in NAc core, and an increase of *PSD-95* in NAc shell, in comparison with vehicle, while non-significant effects on expression of *BDNF* or *PSD95* were evident in PL and IL, or in all punches of animals that were infused with the antagonist non-contingently with reactivation, but in NAc shell where lower *BDNF* levels were evident.

Overall these results indicate the NAc core and shell as key target structures involved in the neuroplastic adaptations mounted by PL cortex upon prazosin infusion and associated with the facilitation of extinction. Interestingly, we found that in NAc core the significative increase in *BDNF* mRNA levels was associated to similar trend in *PSD-95* accordingly to previous reports (Li et al., 2013). This data could be interpreted in light of the different role of the two molecules in neuroplastic mechanisms. While *BDNF* indicates an ongoing neurotrophic activity in neurons, *PSD-95* indicates the morphological result of neuroplasticity, consequence of *BDNF* activity and thus delayed in time. The transcriptional modulation observed demonstrates an active dynamics of neuroplasticity mechanisms induced by PL-infused prazosin in NAc, despite the detection of mRNA rather then protein, allows us only to speculate on the functional direction of the differences observed.

These results, that can not rule out other different neural plasticity effects in cortical functioning in our experimental conditions, indicate that PL (target area of prazosin priming) is likely to drive functional process in subcortical areas that result in neuroplasticity changes strongly related to extinction of Amph-induced CPP.

Indeed, *BDNF* and *PSD95* expression increase was evident only in animals that had extinguished, but not in vehicle matched

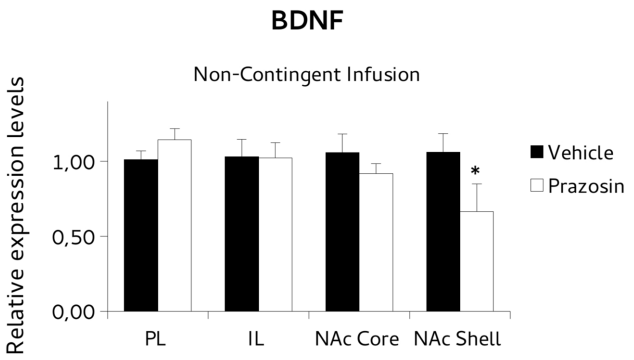

FIGURE 8 | Brain-derived neurotrophic factor (BDNF) mRNA expression in punches of IL and PL cortices, and NAc core and shell subregions, measured after the extinction of amphetamine-induced CPP, in mice that received non-contingent infusion of prazosin or vehicle in PL. The BDNF mRNA expression level (relative expression levels = fold changes over vehicle, normalized to GAPDH gene) was significantly decreased in the NAc shell of Prazosin versus Vehicle-infused mice. $*p < 0.05$.

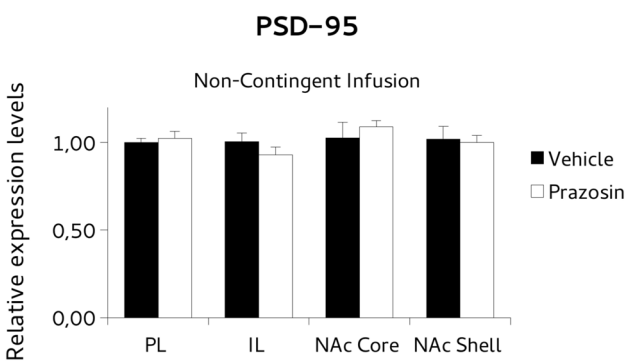

FIGURE 9 | Postsynaptic density protein 95 (PSD-95) mRNA expression in punches of IL and PL cortices, and NAc core and shell subregions, measured after the extinction of amphetamine-induced CPP, in mice that received non-contingent infusion of Prazosin or Vehicle in PL. Non-significant differences were observed in PSD-95 mRNA expression level (relative expression levels = fold changes over vehicle, normalized to GAPDH gene) in either structure.

group or in mice that received prazosin out of the retrieval session, namely non-contingently with exposure with the CPP apparatus and that not extinguished yet preference for Amph paired CS. It worth noting also that these effects were exposure-dependent, a relevant result in the perspective of possible drug assisted behavioral therapy pointed out by our preclinical model.

Moreover behavioral and molecular results are relevant since they point out a possible "priming" effect of the antagonist that suggests a potential therapeutic power (to be further ascertained), given by efficacy of single or sporadic pharmacological (prazosin) treatment with the huge advantage of limiting possible side effects of a repeated treatment.

Note that α1-AR stimulation was reported to induce neuroplasticity (Sawaki et al., 2003; Neverova et al., 2007; McElligott et al., 2010), therefore antagonists should be possibly able to impair neuroplasticity processes induced by receptor activation but is questionable that antagonists themselves produce neuroplastic effects through receptor blockade *per se*.

A body of research of R.S. Duman and his associates has addressed authoritatively the mechanisms of neural plasticity induced by receptor antagonists. They have demonstrated that two antagonists of NMDA or muscarinic receptor, ketamine and scopolamine respectively, provided of clear antidepressant properties, after acute intra-cortical infusion produce increased glutamate transmission that is able to trigger neuroplasticity mechanisms leading to long-lasting behavioral and synaptic changes, also through BDNF. The action of acute (single) administration is transient, and its effects on glutamate depend on blockade of GABA interneurons tonic firing (Duman and Aghajanian, 2012, 2014; Wohleb et al., 2017). Moreover, it has been recently reported that acute ketamine infused in mPFC modulate extinction of conditioned fear (Girgenti et al., 2017).

In these studies neuroplasticity effects occur in cortical areas where drugs are infused, while our present results show neuroplasticity occurring in NAc, although other neural plasticity mechanisms not assessed here cannot be ruled-out. These effects on NAc are consistent with a role of mesolimbic system in modulation of salient stimuli and motivated behavior characterizing CPP to a psychostimulant such that used in our experiments. However, neural plasticity events are likely to originate in the prefrontal cortex and need the animal to be exposed to CS, as is clearly indicated by the results of prazosin infusion non-contingently to the extinction process.

Similarly to what shown for ketamine and scopolamine, we hypothesize that prazosin triggers mechanisms that involves different neurotransmitters. This view is supported by unpublished results obtained in reverse microdialysis experiments showing that prazosin, in CPP extinction procedure, increases dopamine outflow while decreases GABA levels in PL. These effects could spur molecular events, possibly involving glutamate, to promote neural and behavioral plasticity. It is worth noting to mind that a possible increase of prefrontal cortical dopamine by prazosin was hypothesized by Blanc and coworkers a couple of decades ago (Blanc et al., 1994).

Thus it is conceivable that prazosin, possibly altering neural events involving different neurotransmitters in close neurons in PL or in distal neuron of areas connected with PL (e.g., NAc), directly or through trans-neuronal feedback, is able to trigger neuroplastic adaptations such those we observed here. Further study could possibly elucidate this point.

Present results can involve the action of different factors. NE transmission in prefrontal cortex is pivotal in acquisition of motivational properties of different classes of rewarding and aversive stimuli that spur NE release in this area (Ventura et al., 2005, 2007, 2008). Moreover, exposure to appetitive CS can induce prefrontal NE release and the magnitude of this release is positively related to the salience of the US during the training session (Mingote et al., 2004; Ventura et al., 2008). Note that increased prefrontal NE release leads to increase of DA release in the NAc and the paired prefrontal-accumbal catecholamine are crucial in attribution of motivational salience involving highly salient USs (Darracq et al., 1998; Ventura et al., 2003, 2005, 2007).

Therefore, we hypothesize that repeated exposure to the environment paired with Amph was able to increase NE levels in mPFC in control (vehicle) animals, and that this increase contributed to maintain the motivational properties of CS fostering drug-seeking behavior. Conversely, acute intra-PL prazosin blocked the effects of NE release on α1-ARs in animals exposed to Amph conditioned environment. This resulted in blunting the motivational properties of CS and reducing the persistence of CPP in following days, supported by neuroplasticity processes as those observed in the NAc. Moreover, it is well known that repeated non-reinforced exposure to the CS reduces the CR leading to its extinction (Pavlov, 1927; Berman et al., 2003). Accordingly, extinction is conceptualized as a new context-dependent learning that competes with the original learning to control motivation and behavior (Bouton, 2004; McNally, 2014). Therefore, during extinction trials, the original association (Amph-CS) could have undergone a substantial reduction of salience under prazosin action, due to the antagonist-induced impairment of prelimbic NE transmission following exposure to the CS.

Our results point to neuroplasticity effects in NAc, suggesting a prefrontal-accumbal modulation of CS related motivational salience. These effects may be due to prazosin blockade of activation of postsynaptic Gq-coupled α1-ARs and PKC that have been shown to inhibit persistent activity of prefrontal pyramidal neurons (Birnbaum et al., 2004), Therefore, it can be hypothesized that prazosin in PL increases the activity of pyramidal neurons in this area counteracting the hypoactivity of mPFC possibly induced by both Amph administration and conditioned NE release and thus sustaining the inhibitory control over drug-seeking, a view that needs further study to be ascertained.

In addition to this, a possible interplay between PL and IL should be taken into account. Indeed, we have recently shown that NE transmission modulates extinction of Amph-induced CPP through a balance between PL and IL (Latagliata et al., 2016). Therefore, exposure to Amph-paired environment in intra-PL prazosin animals could have shifted the weight of NE in favor of the IL, thus fostering the emergence of extinction trace that would prevail in modulating behavioral outcome. Further experiments could elucidate this point. However, it is worth noting that we observed here changes in BDNF and PSD-95 expression in both accumbal subregions core and shell that are well known to be involved in motivation and in extinction also due to their connections with PL and IL (Bouton, 2004; Miller and Marshall, 2004; Kalivas et al., 2005; Peters et al., 2009; Martín-García et al., 2014; McNally, 2014). Thus, it may be that prefrontal driven modulation of NAc core and shell, resulting in *BDNF* and *PSD-95* expression, promote extinction of US-CS association and/or blunt motivational salience of CS respectively.

Our present results leave a number of points open. Additional specific molecular mechanisms involved in neuroplasticity could be further investigated, to ascertain the effects of acute prazosin in short- and in long-term plasticity in PL. The involvement of other neurotransmitter action, such as dopamine, GABA, glutamate on plasticity and behavior must be assessed. Unveiling the role of different neurotransmitters can help to envisage possible acute therapy based on combined treatment with compounds acting synergistically on different neural mechanisms to spur synaptic and learning processes, possibly at low dosage to minimize side effects. The effects of acute systemic antagonist administration on brain and behavior are to be elucidated and compared with those of intra-cerebral infusion, to assess its therapeutic power.

CONCLUSION

A single pre-reactivation infusion of prazosin in the PL of C57BL/6J mice did not affect expression of Amph-induced CPP the day of infusion, while in subsequent days it produced a clear-cut advance of extinction, an effect depending on contingent exposure to retrieval, since if infused far from or after reactivation it did not affect preference.

BDNF and *PSD-95* expression increase in the NAc Core and Shell, that are part of a prefrontal-accumbal network modulating incentive salience and extinction, was evident only in animals that had extinguished, but not in vehicle matched group or in mice that had received prazosin out of the retrieval session. These neuroplasticity events triggered by intra-PL prazosin could have tagged CS during CPP retrieval, possibly devaluating it or blunting its association with US, point to a mechanism that could account for non-immediately expressed extinction.

Both behavioral and molecular results are relevant since they point, for the first time to our knowledge, to a possible "priming" effect of prazosin that suggests a potential therapeutic power (to be further ascertained) of the antagonist for maladaptive memories, resulting by the efficacy of a single or sporadic pharmacological treatment allowing to avoid possible side effects of repeated drug treatment.

AUTHOR CONTRIBUTIONS

EL and SP-A designed research; EL, GC, and MS performed behavioral experiments; EL, LL, VO, and AR performed RT-PCR; EL, GC, LL, and SP-A analyzed data; EL, LL, and SP-A wrote the paper.

ACKNOWLEDGMENTS

This work was supported by "Ricerca Corrente", Italian Ministry of Health and Ateneo 2015, Sapienza University of Rome.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 23 August 2017
doi: 10.3389/fphar.2017.00566

Verapamil Blocks Scopolamine Enhancement Effect on Memory Consolidation in Passive Avoidance Task in Rats

*Verónica Giménez De Béjar[1,2], María Caballero Bleda[2,3], Natalija Popović[2,3] and Miroljub Popović[2,3]**

[1] Department of Neurology, Hospital Quirónsalud Murcia, Murcia, Spain, [2] Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca, Murcia, Spain, [3] Department of Human Anatomy and Psychobiology, Faculty of Medicine, University of Murcia, Murcia, Spain

Our recent data have indicated that scopolamine, a non-selective muscarinic receptor antagonist, improves memory consolidation, in a passive avoidance task, tested in rats. It has been found that verapamil, a phenylalkylamine class of the L-type voltage-dependent calcium channel antagonist, inhibits [3H] *N*-methyl scopolamine binding to M1 muscarinic receptors. However, there are no data about the effect of verapamil on memory consolidation in the passive avoidance task, in rats. The purpose of the present study was to examine the effects of verapamil (0.5, 1.0, 2.5, 5.0, 10, or 20 mg/kg i.p.) as well as the interaction between scopolamine and verapamil on memory consolidation in the step-through passive avoidance task, in Wistar rats. Our results showed that verapamil (1.0 and 2.5 mg/kg) administered immediately after the acquisition task significantly increased the latency of the passive avoidance response, on the 48 h retested trial, improving memory consolidation. On the other hand, verapamil in a dose of 5 mg/kg, that *per se* does not affect memory consolidation, significantly reversed the memory consolidation improvement induced by scopolamine (1 mg/kg, i.p., administered immediately after verapamil treatment) but did not change the passive avoidance response in rats treated by an ineffective dose of scopolamine (30 mg/kg). In conclusion, the present data suggest that (1) the post-training administration of verapamil, dose-dependently, improves the passive avoidance response; (2) verapamil, in ineffective dose, abolished the improvement of memory consolidation effect of scopolamine; and (3) exists interaction between cholinergic muscarinic receptors and calcium homeostasis-related mechanisms in the consolidation of emotional memory.

Keywords: memory consolidation, passive avoidance, verapamil, scopolamine, rat

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Sean Commins, Maynooth University, Ireland
Thomas Van Groen, University of Alabama at Birmingham, United States

***Correspondence:**
Miroljub Popović
miroljub@um.es

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *29 June 2017*
Accepted: *09 August 2017*
Published: *23 August 2017*

Citation:
Giménez De Béjar V, Caballero Bleda M, Popović N and Popović M (2017) Verapamil Blocks Scopolamine Enhancement Effect on Memory Consolidation in Passive Avoidance Task in Rats. Front. Pharmacol. 8:566. doi: 10.3389/fphar.2017.00566

INTRODUCTION

The influx of Ca^{2++} through the L-type voltage-gated calcium channels (LVGCCs) promotes several molecular processes that are engaged in learning and memory (Singewald et al., 2015; Bas-Orth et al., 2016; Sachser et al., 2016; Michalak and Biala, 2017; Wiera et al., 2017). Age-related memory loss as well as memory impairment in neurodegenerative and psychiatric diseases has been related to the LVGCCs dysfunction with consequent dysregulation of calcium homeostasis

(Barad, 2003; Thibault et al., 2007; Berger and Bartsch, 2014; Yoshimizu et al., 2015; Zanos et al., 2015).

Initially, LVGCCs antagonists, such as benzothiazapines (e.g., diltiazem); dihydropyridines (e.g., amlodipine, felodipine, isradipine, nicardipine, nifedipine, nimodipine, and nisoldipine), diphenylalkylamines (e.g., flunarizine), and phenylalkylamines (e.g., verapamil), were defined as vasodilator, antiarrhythmic, and antianginal agents (Godfraind, 2017). Nowadays, LVGCCs are recognized for the treatment of central nervous system disorders like bipolar disorder (Cipriani et al., 2016), epilepsy (Nicita et al., 2016), and headache (Tfelt-Hansen and Tfelt-Hansen, 2009) with perspective to be also used for the treatment of Parkinson's disease (Stayte and Vissel, 2014; Surmeier et al., 2017), mood disorders (Kabir et al., 2017), and dementia (Nimmrich and Eckert, 2013).

In comparison to other LVGCCs antagonists, verapamil effects on memory formation are highly inconsistent. Its effect is not only dependent on dose, duration of the treatment, and memory trace phase, but also on memory task performance and species. In mice, acute verapamil treatment improves acquisition in passive avoidance and elevated plus maze tasks (Biala et al., 2013; Michalak and Biala, 2017) while does not affect acquisition in conditioned avoidance response, T-maze, and linear maze tasks (Malekar et al., 1999; Quartermain and Garcia de Soria, 2001; Quartermain et al., 2001). It has been found that acute verapamil treatment impairs familiarity discrimination and perirhinal plasticity, in rats (Seoane et al., 2009). Rats exposed for prolonged period to a high dose of verapamil (50 mg/kg) impaired passive avoidance learning, while lower doses applied either acutely or chronically did not affect passive avoidance performance (Lazarova-Bakarova et al., 1997; Lashgari et al., 2006). The chronic verapamil treatment in rats does not modify acquisition but prejudices retention of the radial maze task (Borroni et al., 2000; Woodside et al., 2004).

Memory consolidation is a fragile part of memory traces that occurs immediately after a learning event and lasts for several hours. In this period, the synthesis of plasticity-related proteins and consequent changes in synaptic strength bring to long-term memory storage in the brain (McGaugh, 2000; Kandel, 2001). Previous studies based on pharmacological modulation of cholinergic transmission by scopolamine, non-selective antimuscarinic agent, reported that scopolamine either does not interrupt (Elrod and Buccafusco, 1988; Cruz-Morales et al., 1992; Roldán et al., 1997; Mishima et al., 2001), impairs (Doyle and Regan, 1993; Sigala et al., 1997; Murphy et al., 2001; Hefco et al., 2003; Foley et al., 2004; Gutierres et al., 2012; Popović et al., 2015), or even improves memory consolidation (Popović et al., 2015), in rats tested in the passive avoidance task. The improving effect was only obtained if scopolamine was administered within 6.5 h after learning and when animals were tested 48 h after acquisition (Popović et al., 2015). Systemic post-training treatment with verapamil impairs habituation in rats exposed to the open filed task (Popović et al., 2016), but in mice preserves memory consolidation in the passive avoidance (Quartermain et al., 1993; Masoudian et al., 2015) and improves retention in the linear maze and elevated plus maze tasks (Biala et al., 2013).

It has been suggested by Quartermain et al. (2001) that the lack of consistency in verapamil effects on memory formation could be due to its number of side effects. Verapamil shares a vasodilatory effect with other LVGCCs antagonists, but together with diltiazem expresses depressant effect on heart rate (van Zwieten and Pfaffendorf, 1993; Noll et al., 1998). Moreover, verapamil tends to suppress the activity of sympathetic nervous system (Noll et al., 1998). Besides, verapamil does not block only LVGCCs channels (being Cav1.2 channels more sensitive than the Cav1.3 ones), but also Cav2.1, Cav2.2, Cav2.3, and Cav3.2 channels too (Ishibashi et al., 1995; Cai et al., 1997; Dobrev et al., 1999; Tarabova et al., 2007; Kuryshev et al., 2014). It has been demonstrated that in several brain regions (e.g., cerebral cortex, hippocampus, and hypothalamus), verapamil acts as an antagonist of muscarinic (Baumgold, 1986; Popova et al., 1990), serotoninergic (Taylor and Defeudis, 1984; Adachi and Shoji, 1986; Green et al., 1990; Popova et al., 1991; Shad and Saeed, 2007), dopaminergic (Sitges and Guarneros, 1998), α- and β-adrenergic (Galzin and Langer, 1983; Staneva-Stoytcheva et al., 1990, 1992), and GABAergic receptors (Staneva-Stoytcheva et al., 1991). In contrast to dose-dependent increase of serotonin and dopamine release, verapamil completely abolishes norepinephrine release in rat hippocampal synaptosomes (Sitges and Reyes, 1995). Aside from these actions on neurotransmission, verapamil is a standard P-glycoprotein inhibitor (Bendayan et al., 2002) and small conductance calcium-activated potassium channels (SK channel) antagonist (Tao et al., 2013).

The relationship between calcium homeostasis, cholinergic system activation and learning and memory, remains to be completely elucidated. Our previous studies indicated that acute and chronic verapamil treatment could ameliorate morphological, physiological, cognitive, and non-cognitive behavioral dysfunctions in rats with cholinergic depletion, suggesting possible relations between cholinergic function and calcium metabolism (Popović et al., 1997a,b,c, 1998a,b, 1999, 2006; Caballero-Bleda et al., 2001). Similarly, Sekhar et al. (2016) demonstrated that verapamil improves scopolamine-induced memory impairment in elevated plus maze and novel object recognition tests. Although evidence exists that verapamil, at the subthreshold, ineffective dose, significantly blocks the improving effect of nicotine on memory consolidation in the elevated plus maze task (Biala et al., 2013), there are no data whether verapamil can modify scopolamine effects on memory consolidation. The aim of the present study was to evaluate the effect of verapamil as well as the interaction between cholinergic muscarinic receptors and calcium homeostasis on memory consolidation, in the passive avoidance task, in rats.

MATERIALS AND METHODS

Experimental Animals

Experiments were carried out on male Wistar rats (200–250 g). The animals were housed in standard Makrolon cages on sawdust bedding. They were kept in an air-conditioned room (20 ± 1°C), at 30% humidity, and under a 12 h light/12 h dark cycle (lights

on from 08:00 to 20:00 h). Food and tap water were available *ad libitum*. Before the passive avoidance performance, each rat was handled daily for 5 min during 1 week. The handling and the passive avoidance test were performed between 16:00 h and 20:00 h.

The animal maintenance and experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the guidelines issued by the Spanish Ministry of Agriculture, Fishing and Feeding (Royal Decree 1201/2005 of 21 October 2005). All procedures with animals were approved by the Animal Ethics Committee of the University of Murcia. Efforts were made to minimize animal suffering and the number of animals used.

Drugs

Verapamil and scopolamine hydrobromide were provided by Sigma, St. Louis, MO, United States. Saline solutions of verapamil (0.5, 1, 2.5, 5, 10, or 20 mg/kg), scopolamine hydrobromide (1 mg/kg and 30 mg/kg), or their combination (5 mg/kg of verapamil followed by 1 mg/kg of scopolamine) were administered intraperitoneally. Control animals were treated intraperitoneally with physiological saline at the dose of 1 ml/kg body weight.

Passive Avoidance Test

The passive avoidance testing was done in an automatically operated commercial Passive Avoidance Apparatus (step-through cage 7550; Ugo Basile, Comerio, Italy). The passive avoidance step-through cage was divided into two equal size compartments (insight dimension 22 cm long × 21 cm wide × 22 cm high, each): START (white and illuminated by a 24 V–10 W bulb) and ESCAPE (black and dark). The two compartments are divided by a partition which embodies an automatically operated sliding door at the floor level. On day 1, each rat was exposed to the exploration trial, by placing it in the START chamber (door closed and shock disconnected) and allowed to explore it for 100 s. After that the door was opened, the rat was allowed to enter into the ESCAPE chamber, and when all four paws were in, the automated slide door was closed. The maximum latency to pass from the START to the ESCAPE compartment was set to 60 s. After 10 s, the rat was removed from the ESCAPE compartment and returned to its home cage. On day 2 (acquisition trial), when the rat entered into the ESCAPE compartment, the door was closed and a 1.0 mA shock was delivered for 5 s. Ten seconds later on, the rat was removed from the ESCAPE compartment, drug administered (saline, verapamil, scopolamine, or verapamil followed by scopolamine treatment), and returned to its home cage. According to the latency period to enter into the ESCAPE compartment on day 1 and sensitivity to the shock (vocalization and jumping response) on day 2, the animals were assigned into 11 groups; thus, there were no significant differences between groups. Eight animals were assigned in each tested group. Forty-eight hours after the acquisition trial, the retention trial was carried out. The test was performed in a similar way to the acquisition trial, but no shock was given. The cage catch pan, grid floor, and side walls were cleaned with 70% ethanol before each animal was tested. The most often cut-off times used in the passive avoidance test are 180, 300, and 600 s. In the present study, the cut-off time for the entrance of the rat into the dark compartment was 9 min (maximum time allowed by the used apparatus). The longer cut-off time is when the individual differences become more apparent (Sahgal, 1993). Therefore, for better drug effect discrimination, three cut-off times (180, 300, and 540 s) were analyzed for each animal and group.

Statistical Analysis

The statistical analysis was made using the SPSS 19.0 statistical package. The data are presented as mean ± standard error of the mean (SEM). The effects of verapamil or scopolamine on the step-through latency were analyzed with the one-way ANOVA, followed by the least significant difference (LSD) *post hoc* test. The two-tailed Student's *t*-test for independent samples was used to analyze the effect of combined verapamil and scopolamine treatments. Differences were considered statistically significant if $p < 0.05$.

RESULTS

On the 48 h retention trial of the passive avoidance test, using a maximum cut-off time of 540 s, the one-way ANOVA test showed significant dose-dependent effect of verapamil on the step-through latency ($F_{(6,49)} = 2{,}483$, $p = 0.036$; **Figure 1C**). In animals treated with verapamil at the doses of 1 and 2.5 mg/kg, the step-through latency on the 48 h retention trial was significantly higher in comparison to the groups treated with saline ($p = 0.037$ and $p = 0.012$, respectively), verapamil in the dose of 0.5 ($p = 0.029$ and $p = 0.009$, respectively) and 20 mg/kg ($p = 0.028$ and $p = 0.009$, respectively). Moreover, animals treated with verapamil at the dose of 2.5 mg/kg showed higher step-through latency in comparison to the animals treated with verapamil at the dose of 10 mg/kg ($p = 0.05$). Although employing a 300 s cut-off time, the one-way ANOVA test showed no significant dose-dependent effect of verapamil on the step-through latency ($F_{(6,49)} = 1{,}512$, $p = 0.194$), there were significant differences between animals treated with verapamil in the dose of 2.5 mg/kg and animals treated with saline ($p = 0.043$), verapamil in the doses of 10 and 20 mg/kg ($p = 0.034$ and $p = 0.040$, respectively; **Figure 1B**). Applying a 180 s cut-off time, the one-way ANOVA test showed no significant dose-dependent effect of verapamil on the step-through latency ($F_{(6,49)} = 1{,}132$, $p = 0.358$; **Figure 1A**).

The one-way ANOVA test did not showed significant dose-dependent effect of scopolamine on the step-through latency, utilizing 180, 300, and 540 s cut-off time ($F_{(2,21)} = 0.472$, $p = 0.630$; $F_{(2,21)} = 1{,}074$, $p = 0.360$; and $F_{(2,21)} = 2.995$, $p = 0.72$, respectively) (**Figures 2A–C**). However, when evaluating at 540 s cut-off time, the step-through latency was significantly higher in animals treated with scopolamine in the dose of 1 mg/kg, in comparison to those treated with saline and scopolamine in the dose of 30 mg/kg ($p = 0.045$ and $p = 0.047$, respectively; **Figure 2C**). The animals treated by the combined treatment of scopolamine (1 mg/kg) and verapamil (5 mg/kg) significantly

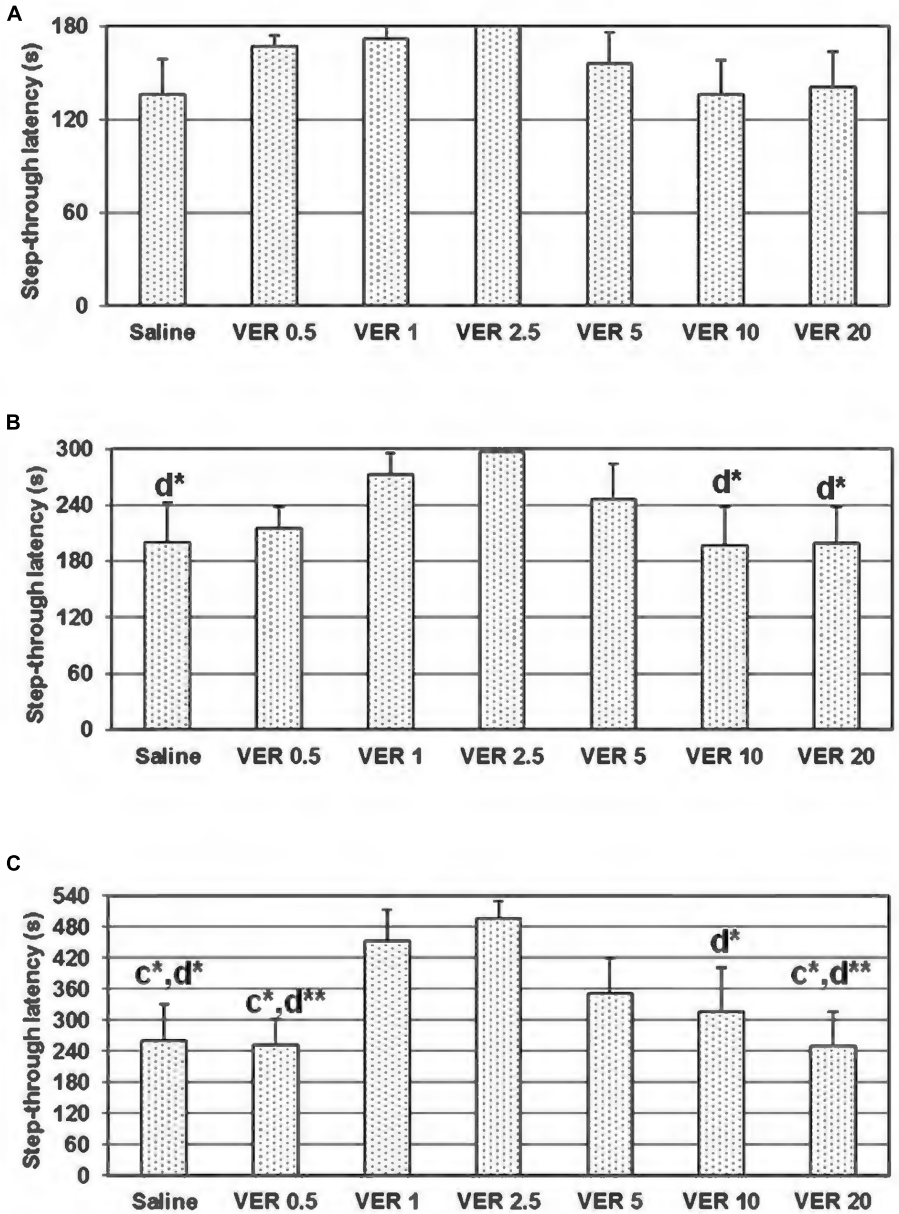

FIGURE 1 | Effect of 0.5, 1, 2.5, 5, 10, and 20 mg/kg of verapamil (VER-0.5, VER-1, VER-2.5, VER-5, VER-10, and VER-20, respectively), administered i.p., immediately after the acquisition trial, on the 48 h retention trial, in the passive avoidance task, in conditions of 180 **(A)**, 300 **(B)**, and 540 s **(C)** cut-off latency. The data are presented as mean ± standard error of the mean (SEM). C-compared to VER-1 and d-compared to VER 2.5; $^{*}p < 0.05$, $^{**}p < 0.01$.

reduced the step-through latency in comparison to the group treated only by scopolamine (1 mg/kg) ($t = 2.219$, $df = 14$, $p = 0.044$; **Figure 2C**).

DISCUSSION

It has been demonstrated that verapamil treatment in the dose of 20 mg/kg does not modify, but that in the dose range from 1 to 10 mg/kg significantly improves consolidation of spatial memory in the linear maze task, in mice (Quartermain et al., 2001). In the elevated plus maze task, only mid-doses used of verapamil (5 and 10 mg/kg) display enhancement effect on spatial memory consolidation, in mice (Biala et al., 2013). The present data indicated that verapamil at the doses of 1 and 2.5 mg/kg, but not at the doses of 0.5, 5, 10, and 20 mg/kg, improved memory consolidation of rats tested in passive avoidance task, confirming the inverted U-shape dose–response curve of its action on memory consolidation. In contrast to the present study, Quartermain et al. (2001) and Masoudian et al. (2015) found that the post-training treatment with verapamil (1, 2, 10, and 20 mg/kg and 1, 2.5, 5, 10, and 20 mg/kg, respectively) did not

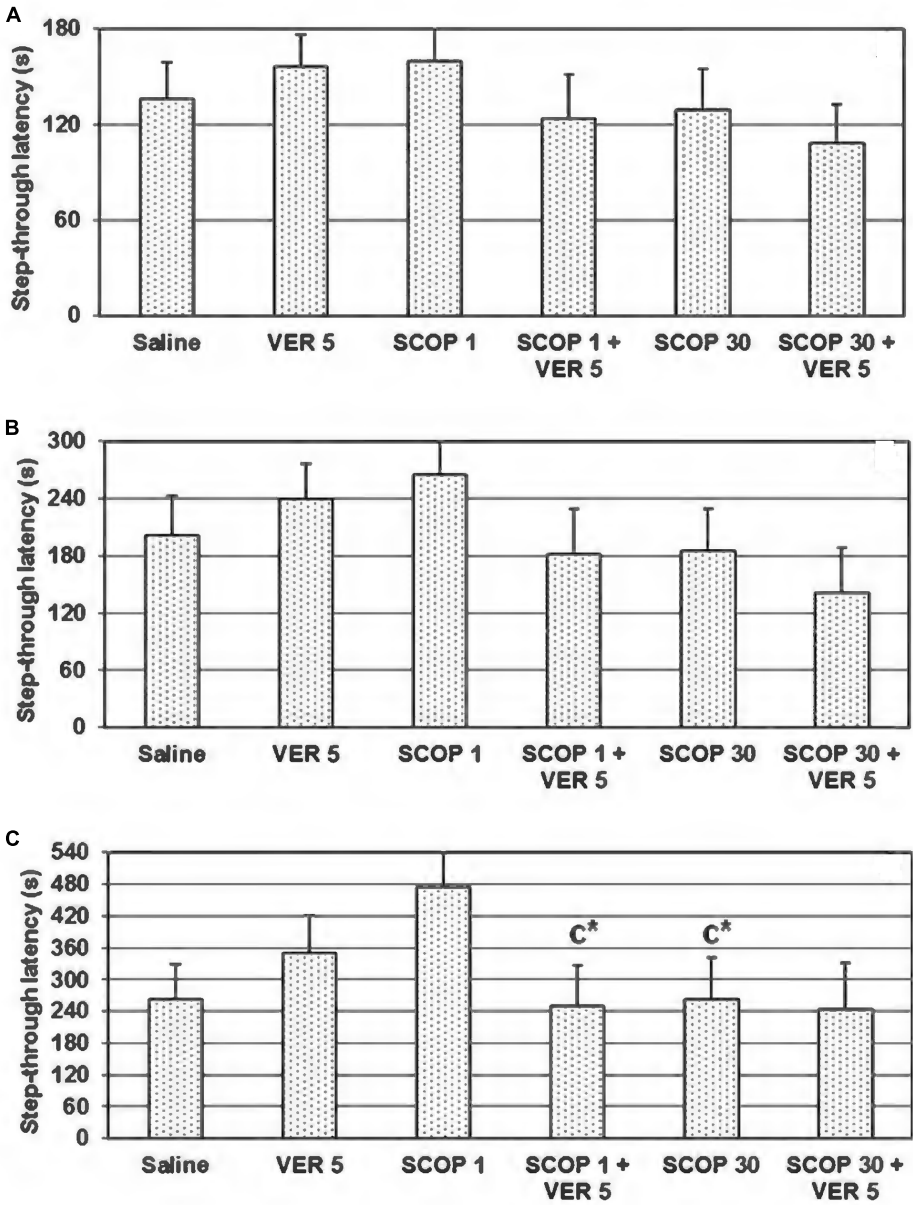

FIGURE 2 | Effect of combined verapamil (VER) 5 mg/kg and scopolamine (SCOP) (1 or 30 mg/kg), administered i.p., immediately after the acquisition trial, on the 48 h retention trial, in the passive avoidance task in conditions of 180 **(A)**, 300 **(B)**, and 540 s **(C)** cut-off latency. Data are presented as mean ± SEM. C-compared to SCOP-1; $*p < 0.05$.

change the retention of the passive avoidance task in mice. The discrepancies between data obtained in passive avoidance studies could be attributed to the species utilized (mice vs. rats), shock intensity (0.1 or 0.5 vs. 1 mA), duration of the shock (1 or 2 vs. 5 s), acquisition–retention interval (24 or 48 h), as well as the duration of the retention trial applied (400 vs. 540 s).

Glick and Zimmerberg (1971) and Rush (1988) showed that scopolamine in higher dose (10–30 mg/kg) impaired memory consolidation in mice tested in the passive avoidance task. The same authors demonstrated that the dose-dependent impairment is related to the duration of the cut-off latency (180, 300, or 600 s), such as longer cut-off latency and lower scopolamine dose are necessary to induce memory impairment. To discard the influence of the cut-off latency time, in the present study we also performed statistical analyses with two shorter frequently used cut-off latency periods (180 and 300 s). Our data suggest that verapamil administered in the dose of 2.5 mg/kg significantly improved memory consolidation in the passive avoidance task, when 300 and 540 s cut-off time were utilized. In contrast, scopolamine in the dose of 1 mg/kg improved memory consolidation when 540 s (Popović et al., 2015), but not when shorter (180 and 300 s) cut-off time was employed.

On the other hand, 30 mg/kg of scopolamine (independent of the cut-off latency time) applied in the present study, as well as 50 mg/kg of it, used by Anagnostaras et al. (1999), did not modify memory consolidation in rats tested in the passive avoidance and fear condition tasks, respectively. These results imply that optimal level of cholinergic neuronal firing, crucial for memory formation, is not only task (Hasselmo and McGaughy, 2004; Hasselmo and Sarter, 2011) but could be also species depending.

The mechanism by which verapamil facilitates or impairs consolidation of emotional memory is still unclear. Findings obtained in Cav1.2 knockout mice argue against the role of the Cav1.2 channels in the consolidation of emotional memory (McKinney et al., 2008). On the other hand, impaired ability of Cav1.3 knockout mice to consolidate contextually conditioned fear but with preserved consolidation in the hidden platform version of the Morris water maze test reveals that the deficits observed in these mice are the result of a disruption of neuronal function within the amygdala (McKinney and Murphy, 2006). Given an inverted U-shape dose–response curve of the verapamil action, Biala et al. (2013) proposed that its action could be due to modulation rather than a complete blockade of LVGCCs (Biala et al., 2013). The facts that: (1) CaV1.2 subtypes are blocked at much lower doses of verapamil in comparison with the CaV1.3 subtypes (Tarabova et al., 2007); (2) higher doses of LVGCCs blockers are required to effectively inhibit brain CaV1.2 and CaV1.3 channels (Helton et al., 2005; Zamponi et al., 2015); and (3) hypotension could improve memory consolidation in the passive avoidance task (Haile et al., 2012), suggest the possibility that outcomes of low doses verapamil treatment, on the passive avoidance consolidation, could be attributed to its effect on the cardiovascular system rather than to the effect on neurons and most likely modulated via CaV1.2 subtypes of LVGCCs.

In view of the facts that verapamil can block SK channel (Tao et al., 2013) and that the blockade of SK2 channels, immediately after the training, enhanced contextual fear memory (Murthy et al., 2015), it could be expected that the effect of verapamil is partially due to the action on these channels, too. On the other side, verapamil expresses both α_1 and α_2 adrenergic receptor blocking activity (Müller and Noack, 1988) and blocking of the α_2 adrenergic receptor subtype improves memory consolidation in the passive avoidance task (Ferry et al., 2015).

Other potential mechanism of verapamil effects on memory consolidation could be related to its antagonistic action on cholinergic receptors. Biala et al. (2013) demonstrated that verapamil, at the subthreshold, ineffective dose (2.5 mg/kg), significantly blocked the improving effect of nicotine (0.035 mg/kg) on memory consolidation, in the elevated plus maze task. Similarly, the present data showed that verapamil, in ineffective dose (5 mg/kg), abolished the improvement of memory consolidation effect of 1 mg/kg of scopolamine but did not change the passive avoidance response when ineffective dose of scopolamine (30 mg/kg) was used. Considering that nicotinic antagonists can block the scopolamine effect of memory formation (Newman and Gold, 2016) and that verapamil inhibited [^{3}H] *N*-methyl scopolamine binding to M1 muscarinic receptors in the rat brain cortex (Baumgold, 1986), further studies are need to determine the cascade of verapamil action on memory consolidation.

CONCLUSION

As far as we know, the present data represent the first demonstration that verapamil dose-dependently improves memory consolidation of the passive avoidance task in rats, and that exists interaction between cholinergic muscarinic receptors and calcium homeostasis, on memory consolidation.

AUTHOR CONTRIBUTIONS

VG, MC, NP, and MP contributed to the design of the study, wrote the protocol, and managed the literature searches; VG, NP, and MP performed the experiments and undertook the statistical analysis; and VG, MC, NP, and MP contributed to drafting the work and have approved the final manuscript.

FUNDING

Funding for this study was provided by the Health Council of Murcia Region, Spain (MC), the Spanish Ministry of Economy and Competitiveness (BFU2014-57516-P; LPL, JLF), and the European Regional Development Fund (EFDR; LPL). The funding source had no further role in the study design; in the collection, analysis, and interpretation of the data; in the writing of the report; or in the decision to submit the paper for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 27 September 2017
doi: 10.3389/fphar.2017.00691

Protective Effects of Sodium (±)-5-Bromo-2-(α-Hydroxypentyl) Benzoate in a Rodent Model of Global Cerebral Ischemia

Yuan Gao[1], Miao Li[1], Yan Wang[1], Zhengqi Li[1], Chenyu Fan[1], Zheng Wang[1], Xinyu Cao[1], Junbiao Chang[2] and Hailing Qiao[1]**

[1] Institute of Clinical Pharmacology, Zhengzhou University, Zhengzhou, China, [2] College of Chemistry and Molecular Engineering, Zhengzhou University, Zhengzhou, China

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***Correspondence:**
Hailing Qiao
qiaohl@zzu.edu.cn
Junbiao Chang
changjunbiao@zzu.edu.cn

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 26 July 2017
Accepted: 15 September 2017
Published: 27 September 2017

Citation:
Gao Y, Li M, Wang Y, Li Z, Fan C, Wang Z, Cao X, Chang J and Qiao H (2017) Protective Effects of Sodium (±)-5-Bromo-2-(α-Hydroxypentyl) Benzoate in a Rodent Model of Global Cerebral Ischemia. Front. Pharmacol. 8:691. doi: 10.3389/fphar.2017.00691

The aim of the current study was to explore the protective effects of sodium (±)-5-bromo-2-(α-hydroxypentyl) benzoate (brand name: brozopine, BZP) in a rat model of global cerebral ischemia. The rat model was established using a modified Winocur's method; close postoperative observation was conducted at all times. Neurological function was detected through prehensile traction and beam-walking test. BZP reduced mortality and prolonged the survival time of rats with global cerebral ischemia, within 24 h. There was a decreased survival rate (60%) in the Model group, while the survival rate of the BZP (3 and 12 mg/kg) remarkably increased the survival rate (to 80 and 90%, respectively), in a dose-dependent manner. Compared with the Model group (survival time: 18.50 h), the administration of BZP (0.75, 3, and 12 mg/kg) prolonged the survival time (to 20.38, 21.85, and 23.90 h, respectively), particularly in BZP 12 mg/kg group ($P < 0.05$). Additionally, the BZP (12 mg/kg) group exhibited an improvement in their motor function ($P < 0.05$). The BZP groups (0.75, 3, and 12 mg/kg) displayed significantly reduced necrosis and the percentage of apoptotic cells ($P < 0.05$ and $P < 0.01$, respectively). Compared with Model group, BZP (0.75, 3, and 12 mg/kg) increased the NeuN optical density values ($P < 0.01$). Rats with global ischemia had a high expression of Cyt-c, caspase-3, and the Bax/Bcl-2 ratio compared with sham group ($P < 0.01$). BZP (0.75, 3, and 12 mg/kg), however, reduced the expression of Cyt-c, caspase-3, and the Bax/Bcl-2 ratio, in a dose-dependent manner ($P < 0.01$). There was low expression of p-Akt and PI3K in Model group, compared with the sham group ($P < 0.01$). Meanwhile, BZP (0.75, 3, and 12 mg/kg) increased the expression of p-Akt and PI3K in a dose-dependent manner ($P < 0.01$). We also found the expression of Cyt-c, caspase-3, Bax/Bcl-2 ratio, PI3K, p-Akt, and comprehensive score were directly related. In conclusion, BZP had therapeutic potential and prevented stroke in rat model of global cerebral ischemia. The underlying mechanisms may be related to the inhibition of apoptosis and activation of the survival-signaling-pathway.

Keywords: brozopine, global cerebral ischemic rat, neurological function, apoptosis, survival

INTRODUCTION

Sodium (±)-5-bromo-2-(α-hydroxypentyl) benzoate (BZP), which is derived from 1-3-*n*-butylphthalide (NBP), has a chemical structure that is similar to aspirin. NBP was developed as an anti-cerebral ischemic agent in 2002, in China. Although it was widely used, with good clinic results, increasing reports of adverse reactions such as coagulopathy, gastrointestinal irritation, and liver dysfunction, led to its discontinuation. We designed and synthesized a series of NBP derivatives. The activities of all compounds have been evaluated *in vitro* (Wang et al., 2010). Our previous studies demonstrated that 3-butyl-6-bromo-1(3H)-isobenzofuranone (Br-NBP) had anti-hydrogen peroxide-induced damage in PC12 cells and anti-platelet aggregation effect *in vitro* or *in vivo* on rats (Gao et al., 2010; Ma et al., 2012). Based on our previous findings, BZP played a neuroprotective role against focal cerebral ischemia-reperfusion injury in rats, via anti-apoptosis and anti-inflammation mechanisms, and the promotion of synaptic plasticity (unpublished data). Currently, BZP is used in Phase I clinical trials with encouraging efficacy results. The preventive and therapeutic effects of BZP on a rat model of global ischemia injury have not yet been studied. In this context, we investigated the modulatory effects of BZP on rats following global ischemia injury.

Ischemia injury of brain tissue triggers many cellular complex mechanisms, including excitotoxicity, depolarization, oxidative stress, inflammation, and apoptosis, in addition to many other physiological and pathological processes (Ayuso et al., 2017; de la Tremblaye et al., 2017). Apoptosis is one of the major pathways associated with the activation of a genetic program in which apoptosis effector genes promote cell death, while repressor genes enhance cell survival in cerebral ischemia injury (Yin et al., 2017). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining detects DNA fragmentation, which is one of the hallmarks of apoptosis. Two important groups of proteins in the apoptotic cascades are members of the B-cell lymphoma-2 (Bcl-2) family and classes of caspases (Kim et al., 2017). The Bcl-2 family can be classified into the following functionally distinct groups: anti-apoptotic proteins and pro-apoptotic proteins. Bcl-2, an anti-apoptotic protein, regulates apoptotic pathways and protects against cell death. Bcl-2-associated X protein (Bax), a pro-apoptotic protein of the Bcl-2 family that is highly and selectively during apoptosis, promotes cell death (Pena-Blanco and Garcia-Saez, 2017). Caspase-3 is one of the key executors of apoptosis, and the activation of caspase-3 is implicated in apoptotic neuronal cell death in animal models of stroke (Hwang et al., 2013). The rapid translocation of Bax during cerebral ischemia activates the release of cytochrome c (Cyt-c) from the mitochondria, which activates the caspase cascade, leading to apoptosis. The phosphatidylinositol-3 kinase (PI3K)/Akt pathway is a central mediator in signal transduction pathways involved in cell growth, cell survival, and metabolism. It plays a pivotal role in the defense against various neuronal-damaging insults (Wang et al., 2014; Ju et al., 2015; Yang et al., 2015).

Abbreviations: ASA, aspirin; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-2; Br-NBP, 3-butyl-6-bromo-1(3H)-isobenzofuranone; BZP, sodium (±)-5-bromo-2-(α-hydroxypentyl) benzoate, brozopine; Cyt-c, cytochrome c; DNA, deoxyribonucleic acid; HE, hematoxylin and eosin; NBP, 1-3-*n*-butylphthalide; NBP-K, potassium 2-(1-hydroxypentyl)-benzoate; NeuN, neuronal nuclei; NF-κB, nuclear factor kappa B; PI3K, phosphatidylinositol-3 kinase; SD, Sprague Dawley; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; 2VO, two-vessel occlusion; 6VO, six-vessel occlusion.

MATERIALS AND METHODS

Chemicals

BZP and Br-NBP were synthesized at the Department of Chemistry, Zhengzhou University. The purities of the compounds were 99.4 and 99.8%, respectively. Potassium 2-(1-hydroxypentyl)-benzoate (NBP-K) was purchased from the National Institutes for Food and Drug Control, China. Hematoxylin and eosin (HE) were purchased from Beijing Zhongshan Co., China. Anti-NeuN antibody was purchased from Millipore Co., United States. Mouse monoclonal antibody to Bax, rabbit polyclonal antibody to Bcl-2, rabbit monoclonal antibody to caspase-3, rabbit anti-mouse immunohistochemistry kits to Cyt-c, rabbit polyclonal antibody to PI3K, rabbit polyclonal antibody to p-Akt and TUNEL kits were all purchased from Wuhan Boster Biological Technology Co., China.

Animals

A total of 70 male Sprague Dawley rats (7 weeks old, weighing 220–300 g) were purchased from the Laboratory Animal Center of Henan province. The animals were housed under controlled environmental conditions (lights on from 6:00 am to 6:00 pm, temperature: 24–26°C, relative humidity: 50–60%) and allowed access to a commercial rat chow and tap water *ad libitum*. The animals were allowed to adapt to the environment for at least 1 week. The rats were fasted 12 h prior to the experiments. All animal experiments were approved by the institutional guidelines of the Experimental Animal Center of the Chinese Academy of Medical Science (certification number: SCXK 2010-0002).

Global Ischemia Model

The rats were anesthetized using 10% chloral hydrate, fixed on the constant temperature mouse board, and the body temperature was maintained at 36.5∼37.5°C. Modified Winocur's (Winocur et al., 2013) two vessel blocking method of acute global ischemia model (two-vessel occlusion, 2VO), which were established by permanent ligation of bilateral common, internal, and external carotid arteries (six-vessel occlusion, 6VO) (Li et al., 2015). Sham animals received only separate exposure bilateral common carotid artery, external carotid artery, internal carotid artery, which were not ligated or cut.

Experimental Setup

Once the ischemic model was established, the rats were divided into the following seven groups: sham group, model group, BZP-treated group (0.75, 3, and 12 mg/kg/day), Br-NBP (10.5 mg/kg/day), and NBP-K group (9.6 mg/kg/day, $n = 10$). The sham group and model group received daily intravenous

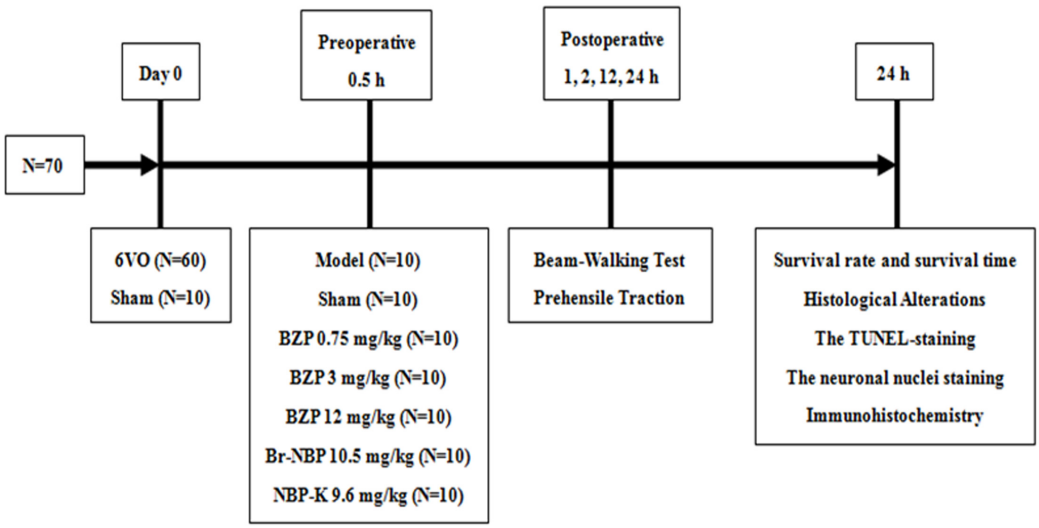

FIGURE 1 | The study design.

doses of the same amount of normal saline and other groups were administered the corresponding drug in the same way. Among these groups, BZP 12 mg/kg, Br-NBP 10.5 mg/kg, and NBP-K 9.6 mg/kg were equimolar doses. According to the design of the study, as presented in **Figure 1**.

Mortality

The postoperative behavioral performances and number of deaths were observed and recorded. Following death, the rat's brain tissues was analyzed for the relevant indicators so that the ischemic injuries could be described quantitatively.

Behavioral Assessment

The behavioral assessment was performed under standard laboratory conditions (temperature: 22–24°C and relative humidity: 45–50%) by a research who was blinded to the rats' experimental groups after 1, 4, 12, and 24 h post-operation.

Beam-Walking Test (Urakawa et al., 2007)

Rats were trained to walk on a wooden beam (2.5 cm × 2.5 cm × 80 cm), which was elevated 60 cm above the floor, to reach their home cage. Their performance was evaluated using a modified scale: score 0, the rat traversed the beam with no foot slip; score 1, the rat traversed while grasping the lateral side of the beam; score 2, the rat showed walking disability on the beam but could traverse it; score 3, the rat took a considerable amount of time to traverse the beam due to difficulty walking; score 4, the rat was unable to traverse the beam; score 5, the rat displayed difficulty in moving the body or any limb on the beam; score 6, the rat was unable to stay on the beam for 10 s.

Prehensile Traction Test (Hattori et al., 2000)

Rats were trained to grab a wire (with a diameter and the length 0.15 and 100 cm, respectively). The wire was elevated 70 cm above the floor and placed over a 5-cm-thick foam pad in case the rats fell. After placing the front paws on the wire, we recorded the time latency until the rat lost hold of the wire. The performance was evaluated on a four-grade score: score 0, hanging on the wire over 5 s and the hind legs placed on the ropes; score 1, hanging on the wire over 5 s; score 2, hanging on the wire for 3–4 s; score 3, hanging on the wire for 0–2 s.

Immunohistochemistry

Following the behavioral assessment test, the rats' brains were removed and immersed in 4% paraformaldehyde with 1 M phosphate buffer, for 48 h. Therefore it was embedded in paraffin, and five groups of 4- to 6-μm-thick sections were prepared for immunostaining and incubated with primary antibodies against Cyt-c, caspase-3, Bax/Bcl-2, p-Akt, or PI3K, followed by incubation with the appropriate secondary antibody.

Statistical Analysis

Graphs were generated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, United States). The Student's unpaired *t*-test, and one-way analysis of variance with Dunnett's *post hoc* test were performed to statistical analyze of the data. Statistical significance was set at criterion $P < 0.05$. The data are presented as the mean ± standard deviation.

RESULTS

BZP Increased the Survival Rate, Prolonged the Survival Time in a Rat of Global Cerebral Ischemia within 24 h

The global cerebral ischemia model was established using the modified Winocur's method on rats. Close postoperative

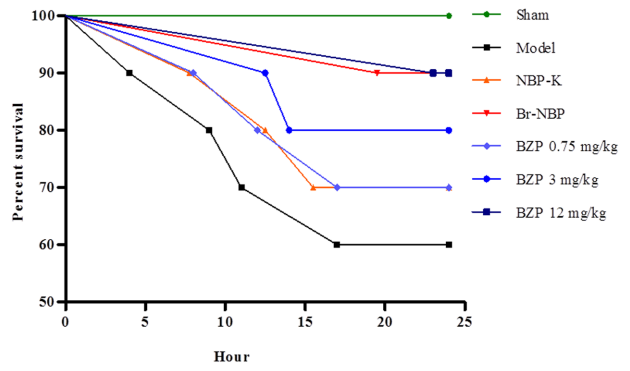

FIGURE 2 | The effect of BZP, within 24 h, on survival rate and survival time in a rat model of global cerebral ischemia. The *x*-axis represents the hour of administration.

observation was maintained at all times. As shown in **Figure 2**, 24 h later, there was a decreased survival rate (60%) in the Model group, while the survival rate of the BZP (3 and 12 mg/kg) remarkably increased the survival rate (to 80 and 90%, respectively), in a dose-dependent manner. Compared with the model group (survival time: 18.50 h), the administration of BZP (0.75, 3, and 12 mg/kg) prolonged the survival time (to 20.38, 21.85, and 23.90 h, respectively), particularly in BZP 12 mg/kg group ($P < 0.05$). These results demonstrated that BZP increased the survival rate and prolonged the survival time in rats with global ischemia.

Behavioral Testing

Following BZP administration, the rat's prehensile traction and beam-walking ability were assessed to directly investigate the effect of BZP on functional recovery. Compared with the sham group, which exhibited an intact performance, the cerebral lesions in the rats with global cerebral ischemia caused behavioral deficits, as evidenced by a reduced time on the beam in the beam-walk test. In contrast, rats in the BZP groups remained on the beam for longer, compared with the Model group. Moreover, in the prehensile traction test, compared with the Model group, rats that were treated with various doses of BZP spent significantly more time on the rope an increase was observed in rats treated with different doses of BZP. There were significant differences in the time spent on the rope ($P < 0.05$). Additionally, the BZP (12 mg/kg) group exhibited an improvement in their motor function, which was more prevalent than in the NBP-K (9.6 mg/kg) and Br-NBP (10.5 mg/kg) groups. A significant group effect in the grip strength was observed at hours 4, 12, and 24, while rats treated with BZP (0.75, 3, and 12 mg/kg) achieved significantly lower scores than the other experimental groups (**Figure 3**).

Histological Alterations

HE staining was performed at 24 h after BZP administration to observe its effect on the morphology of hippocampal neurons. As shown in **Figure 4**, typical neuropathological changes, including neuronal loss and nucleus shrinkage or disappearance were observed in the CA1 of the hippocampus in Model group. Further, there was a reduction in the density of healthy neuron cells in the CA1 region in the Model group compared with the sham group ($P < 0.01$). The BZP groups (0.75, 3, and 12 mg/kg) displayed significantly reduced necrosis ($P < 0.05$ and $P < 0.01$, respectively).

BZP Inhibited Neuronal Apoptosis in a Rat Model of Global Ischemia

TUNEL staining reveals apoptosis-positive cells, which have the following features: cell body shrinkage, nuclear pyknosis, nuclear fragmentation, and the formation apoptotic bodies. In comparison with Model group, as shown in **Figure 5A**, the percentage of apoptotic cells had significantly decreased with BZP (0.75, 3, and 12 mg/kg; $P < 0.01$). In addition, NeuN-expressing mature neurons were absent in the brain tissue after the induction of global ischemia. Compared with model group, as shown in **Figure 5B**, BZP (0.75, 3, and 12 mg/kg) increased the NeuN optical density values ($P < 0.01$). BZP (12 mg/kg) was better than NBP-K group ($P < 0.01$) and Br-NBP group ($P < 0.01$).

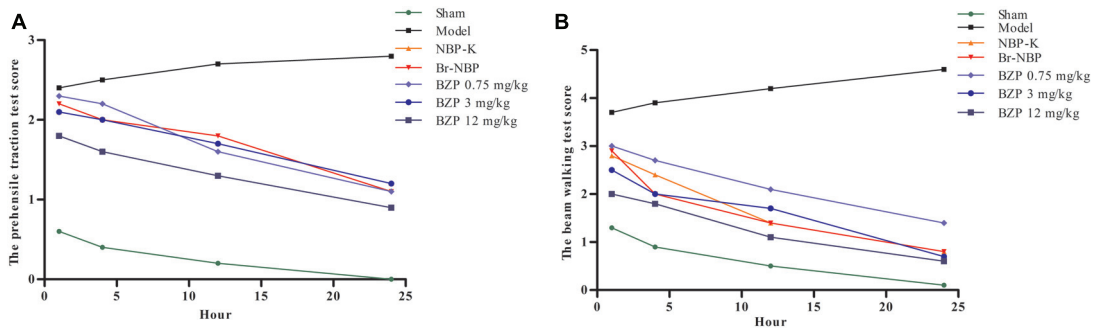

FIGURE 3 | The neurological deficits score on pre-ischemic treatment with BZP in rats with global ischemia. **(A)** Prehensile traction test. **(B)** Beam-walking test. The *x*-axis represents the hour of administration, while the *y*-axis represent the prehensile traction score and beam-walking score. N* is the number of survival animals after 24 h.

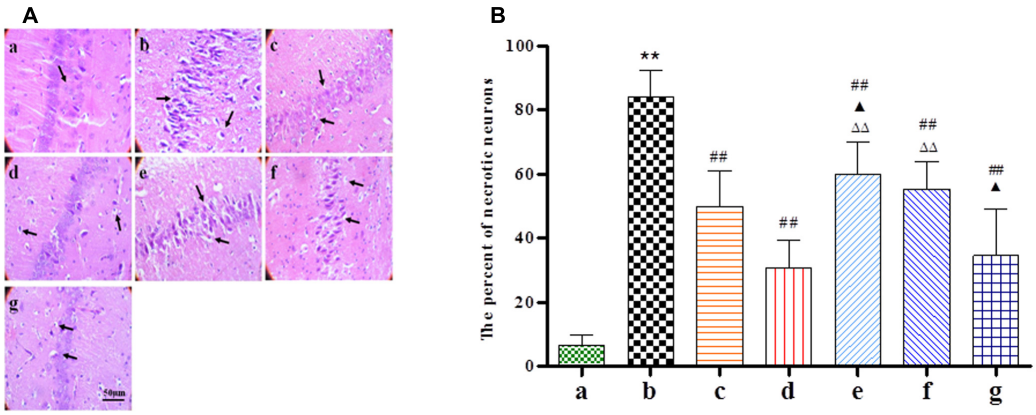

FIGURE 4 | The effect of BZP on morphologic changes in the CA1 region of the hippocampus in rats with global ischemia. The total number of necrosis cells was counted in five distinct areas of the hippocampus under the microscopic field (magnification ×200) in each group. **(A,B)** The different groups are as follows: (a) Sham, (b) Model group, (c) NBP-K (9.6 mg/kg), (d) Br-NBP (10.5 mg/kg), and (e–g) BZP (0.75, 3, and 12 mg/kg, respectively). Data were presented as mean ± standard deviation. A one-way analysis of variance was used to determine if the differences between the groups were statistically significant. $^{**}P < 0.01$ *vs* Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Model group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs* NBP-K group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ *vs* Br-NBP group. Arrows show that nerve cells sparse, irregular, even disappeared, nucleus shrinkage with neuronal necrosis.

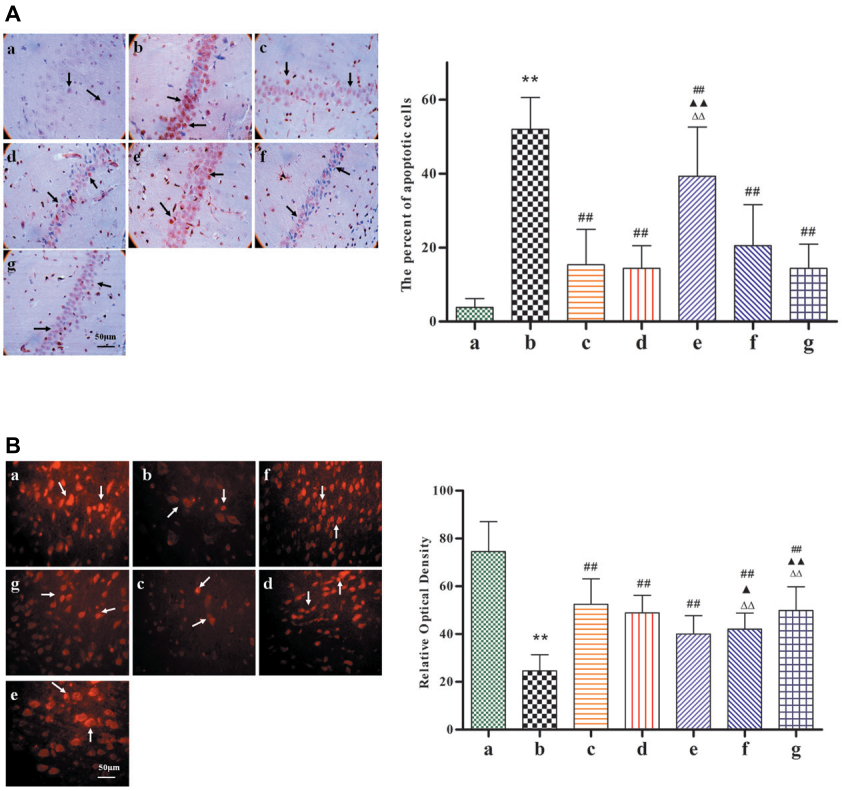

FIGURE 5 | Images of the expression of neuronal nuclei and apoptosis, following pretreatment with BZP, in ischemia-induced rats. Total number of apoptotic cells and neuronal nuclei was counted in five distinct areas of the ischemic penumbra under a microscopic field (magnification × 200), in each group. **(A)** TUNEL-staining. **(B)** Neuronal nuclei staining. The different groups are as follows: (a) Sham, (b) Model group, (c) NBP-K (9.6 mg/kg), (d) Br-NBP (10.5 mg/kg), and (e–g) BZP (0.75, 3, and 12 mg/kg, respectively). Data were presented as mean ± standard deviation. A one-way analysis of variance was used to determine if the differences were statistically significant. $^{**}P < 0.01$ *vs* Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Model group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs* NBP-K group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ *vs* Br-NBP group. Arrows in **(A)** show that cell body shrinkage, nuclear pyknosis, nuclear fragmentation, and the formation apoptotic bodies. Arrows in **(B)** show that mature neurons loss.

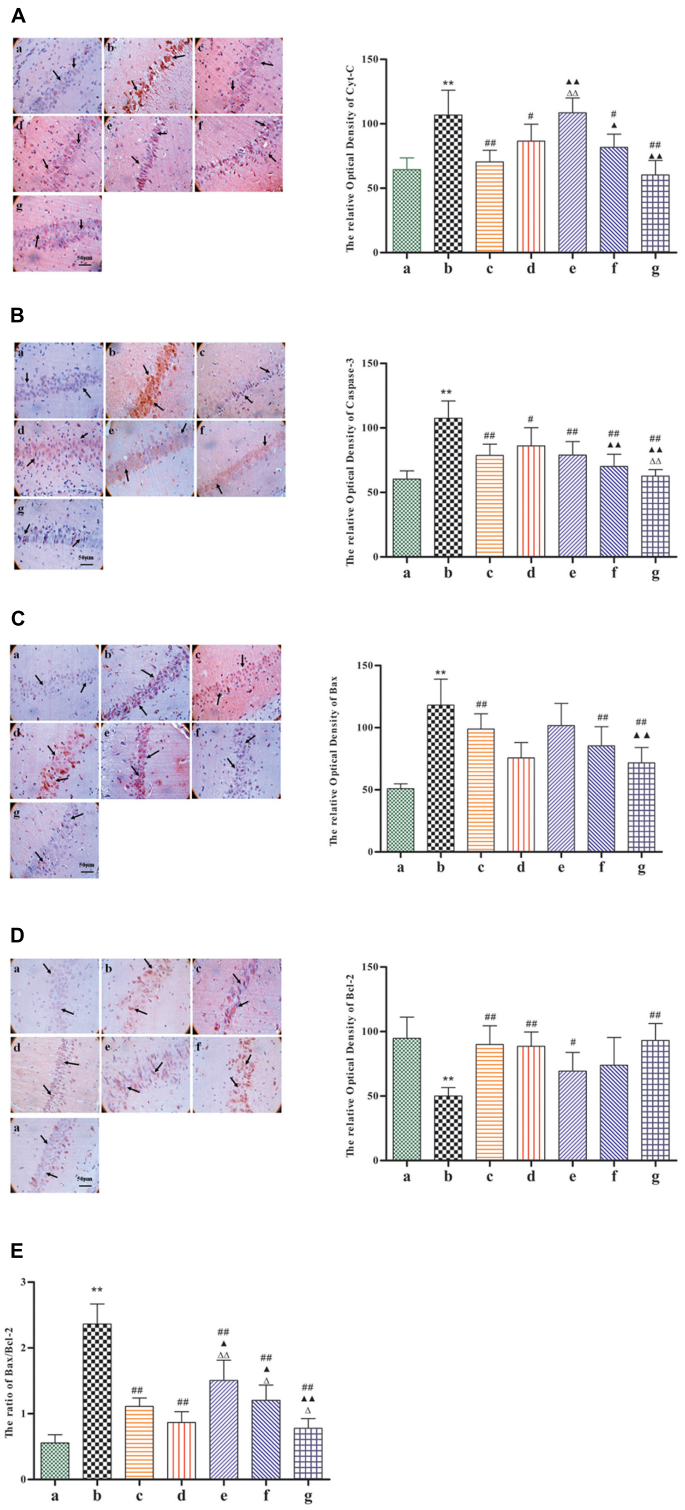

FIGURE 6 | Immunohistochemical staining analysis of Cyt-c, caspase-3, the Bax, and Bcl-2 ratio in the CA1 hippocampal region in rats with global cerebral ischemia, after pretreatment with BZP, NBP-k, or Br-NBP. The total number of positive cells was counted in five distinct areas of the ischemic penumbra under the microscopic field (magnification ×200) in each group. **(A)** Cyt-c. **(B)** Caspase-3. **(C)** Bax. **(D)** Bcl-2. **(E)** The Bax/Bcl-2 ratio. The different groups are as follows: (a) Sham, (b) Model group, (c) NBP-K (9.6 mg/kg), (d) Br-NBP (10.5 mg/kg), and (e–g) BZP (0.75, 3, and 12 mg/kg, respectively). Data were presented as mean ± standard deviation. A one-way analysis of variance was used to determine if the differences between groups were statistically significant. $^{**}P < 0.01$ *vs* Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Model group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs* NBP-K group; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ *vs* Br-NBP group. Arrows show that the expression of Cyt-c, caspase-3, Bax, Bcl-2, p-Akt and PI3K positive cell.

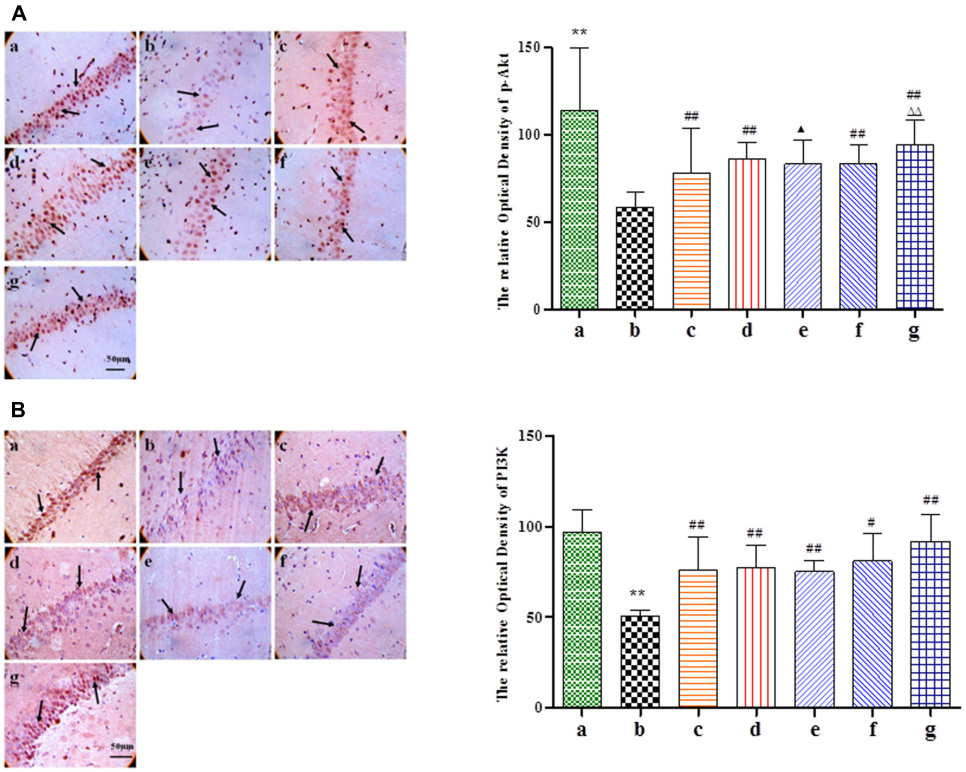

FIGURE 7 | Immunohistochemical staining analysis of p-Akt and PI3K in the CA1 hippocampus region of rats with global cerebral ischemia, after pretreatment with BZP, NBP-K, or Br-NBP. The total number of positive cells was counted in five distinct areas of ischemic penumbra under the microscopic field (magnification ×200) in each group. **(A)** p-Akt. **(B)** PI3K. The groups were as follows: (a) Sham, (b) Model group, (c) NBP-K (9.6 mg/kg), (d) Br-NBP (10.5 mg/kg), and (e–g) BZP (0.75, 3, and 12 mg/kg, respectively). Data were presented as mean ± standard deviation. A one-way analysis of variance was used to determine if the differences between the groups were statistically significant. $^{**}P < 0.01$ *vs* Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* Model group; $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$ *vs* NBP-K group; $^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$ *vs* Br-NBP group. Arrows show that the expression of Cyt-c, caspase-3, Bax, Bcl-2, p-Akt and PI3K positive cell.

BZP Decreased the Expression of Cyt-c, Caspase-3, the Ratio of Bax/Bcl-2, and Elevated the Level of p-Akt and PI3K in the CA1 Hippocampal Region in Rats with Global Ischemia Injury

Given its neuroprotective effects, we further investigated the effect of BZP on the mitochondrial signaling pathway following global cerebral ischemia. The activation of genes for apoptosis factors promotes cell death, while repressor genes enhance cell survival. As shown in **Figure 6**, rats with global ischemia had a high expression of Cyt-c, caspase-3, and the Bax/Bcl-2 ratio compared with sham group ($P < 0.01$). BZP (3, 0.75, and 12 mg/kg), however, reduced the expression of Cyt-c, caspase-3, and the Bax/Bcl-2 ratio, in a dose-dependent manner ($P < 0.01$). In addition, BZP 12 mg/kg had a better effect on the expression of Cyt-c, caspase-3, and the Bax/Bcl-2 ratio than NBP-K 9.6 mg/kg ($P < 0.05$, $P < 0.01$) and Br-NBP 10.5 mg/kg ($P < 0.05$, $P < 0.01$). As shown in **Figure 7**, there was low expression of p-Akt and PI3K in Model group, compared with the sham group ($P < 0.01$). Meanwhile, BZP (3, 0.75, and 12 mg/kg) increased the expression of p-Akt and PI3K in a dose-dependent manner ($P < 0.01$).

Significant Correlation between the Expression of Apoptosis-Related Factors and Comprehensive Score in Rats with Global Ischemia

We performed a correlation analysis between the expression of Cyt-c, caspase-3, Bax/Bcl-2 ratio, PI3K, p-Akt, and comprehensive score in the Model and BZP (0.75, 3, and 12 mg/kg) groups. The comprehensive score was calculated by adding the prehensile-traction score to the beam-walking score. As shown in **Figure 8**, the expression of Cyt-c, caspase-3, Bax/Bcl-2 ratio, PI3K, p-Akt, and comprehensive score were directly related. Thus, a higher expression of Cyt-c, caspase-3, and Bax/Bcl-2, was associated with a lower the expression of PI3K and p-Akt and the more severe neurological deficit.

DISCUSSION

Although BZP was distributed in tissue from both the normal and global cerebral ischemia brains, it was particularly highly concentrated in the latter, where it was mostly metabolized into Br-NBP (data not shown). Interestingly, Br-NBP was an active

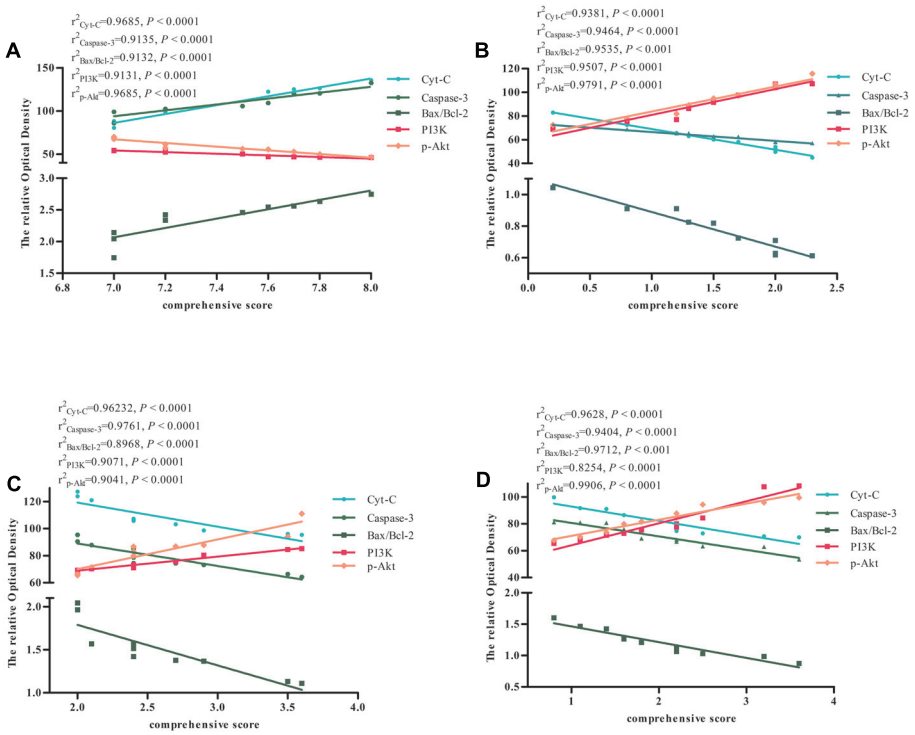

FIGURE 8 | The correlation between the expression of apoptosis-related factors and comprehensive score in Model and BZP (0.75, 3, and 12 mg/kg) groups in rats with global ischemia. Correlation between the expression of apoptosis-related factors and comprehensive score in **(A)** Model group, **(B)** BZP 12 mg/kg group, **(C)** BZP 3 mg/kg group, and **(D)** BZP 0.75 mg/kg group. r^2, correlation coefficient of the expression of apoptosis-related factors and comprehensive score. Statistical significant denoted by $P < 0.001$.

metabolite, with anti-oxidant stress and anti-platelet aggregation effects. Therefore, Br-NBP was an active metabolic compound that was closely observed. NBP-K was regarded as the pro-drug of NBP and NBP-K was used as a positive control drug in the current study. The anti-ischemic action of BZP was 4–16 times that of Br-NBP or NBP-K, at the equivalent molar dose. Thus, we speculated that the neuroprotective effect of BZP depended on itself and Br-NBP. The potency of BZP was greater than Br-NBP, implying that it had a new chemical structure and was different from NBP-K and Br-NBP. The present study demonstrated the neuroprotective effect of BZP against global cerebral ischemia injury.

A model of global ischemia should closely mimic the main clinical symptoms of the disease. Compared with the traditional 2VO model, the neurological deficit, motor coordination, and pathological damage were more serious in the 6VO model. Therefore, the 6VO model was more suitable as a rat model of global cerebral ischemia, for the current study, in comparison to the 2VO method. The results also highlighted that the administration of BZP (0.75, 3, and 12 mg/kg) and Br-NBP decreased the mortality and prolonged the survival time, particularly at higher doses.

Since global ischemic injury had destroyed the sensory and motor centers of the rat model of the disease, assessing behavioral function recovery allowed the effect of the BZP treatment to be evaluated. The beam-walking test is a common method used to gauge the recovery of sensory and motor function. The prehensile traction test is used to measure the ability of the forepaw and forelimb in rats. In our data, at 24 h, the rats could walk properly in the sham group. Compared with the Model group, the scores of rats in the treatment groups were lower and gradually, decreased over time. This suggested that the motor function was gradually restored. The active function of the rat upper limbs and balance were improved by a high dose of BZP was superior to NBP-K or Br-NBP.

Treatment with BZP also inhibited the mitochondrial apoptotic pathway, and markedly reduced ischemia-induced neuronal apoptotic cells within penumbra, 24 h after global cerebral ischemic injury. Experimental and clinical evidence has demonstrated that cerebral ischemia disrupts the blood–brain barrier permeability (Beker et al., 2015; Hong et al., 2015). The present study provided evidence to confirm that BZP exerted its neuroprotective effects through the inhibition of the mitochondrial apoptotic pathway after transient global cerebral ischemia. Additionally, BZP reduced the number of TUNEL-positive cells and apoptotic bodies, and even enhanced the level of anti-apoptotic protein. Apoptosis is considered the prominent form of neuronal death in the penumbra in stroke (Cheng et al., 2015). The penumbra, which is an area of partially preserved energy metabolism, represents ischemic brain tissue that is functionally impaired, but where the injury is potentially reversible. When considering therapeutic strategies for ischemic

stroke, the rescue of the penumbra is a crucial consideration (Chelluboina et al., 2015). There is increasing evidence that the penumbra area of nuclear factor kappa B (NF-κB) activity significantly overlaps with tumor necrosis factor alpha (TNF-α) overproduction and apoptosis. The NF-κB activity and enhanced TNF-α expression could be attributed to an apoptotic pathway following global cerebral ischemia. Apoptosis, which is mediated by mitochondrial disturbances, is a major cause of cellular damage in the ischemic penumbra. The mitochondrial release of apoptogenic factors drives neuronal cell death during cerebral ischemia. Proteins in the proapoptotic Bcl-2 family, such as Bax and BAK, reside in the mitochondrial outer membrane and constituted a gateway to the apoptotic process. Bax and BAK are also located at the endoplasmic reticulum, where they regulated calcium fluxes. Bax, which is homologous to Bcl-2, has antagonistic actions to the latter. Thus, the ratio of these proteins is an index to evaluate the level of cell apoptosis. High levels of calcium in the mitochondria disrupts the mitochondrial outer membrane by increasing permeability transition and promoting Cyt-c release, which acts as a trigger for neuronal apoptosis. Caspases become subsequently activated, resulting in apoptosis (Lazarovici et al., 2012; Li et al., 2012; Jayakumar et al., 2013; Bradley et al., 2014). Caspase-3, in particular, causes degradation of the cytoskeleton, DNA fragmentation, and eventually, cell death. In contrast, anti-apoptotic mediators such as Bcl-2 and Bcl-xL increase cell survival after ischemia by preserving mitochondrial membrane integrity (Chen et al., 2015). The results showed that BZP significantly reduced the expression of Cyt-c and caspase-3 in the ischemic penumbra, while decreasing the ratio of Bax/Bcl-2 in rats with global cerebral ischemia. The strong positive correlation between the changes of NF-κB and other inflammatory proteins corroborated with and validated previous findings. However, we currently do not know why the extent of the correlation between BZP dosages and various inflammatory proteins differ. Further research on detailed anti-inflammatory effects of BZP is currently in progress. Apoptosis is associated with the activation of a genetic program in which apoptosis effector genes promote cell death, while repressor genes enhance cell survival. It has been proven that, in a variety of organisms, the inhibition of the PI3K/p-Akt signaling pathway decreases the rate of cell metabolism, which delays cell senescence (Abdel-Aleem et al., 2016). The inhibition of cell apoptosis is initiated by the activation of PI3K, which activates the upstream kinase, Akt is necessary for the survival of nerve growth factor-dependent sympathetic neurons, which also maintains or enhances the survival of neurons (Crowder and Freeman, 1998). p-Akt can also inhibit the activation of caspase-9. In addition, p-Akt can enter the nucleus and inhibit the phosphorylation of the Forkhead family, thereby blocking the Fas apoptosis pathway (Murphy, 2004; Song et al., 2005). In the current study, BZP increased the expression of PI3K and p-Akt, suggesting that it inhibited apoptosis in rats with global ischemia injury by activating PI3K/p-Akt signaling pathway.

CONCLUSION

BZP could be an effective the treatment and prevention of global ischemic stroke. The results of the current study suggest that BZP elicits neuroprotective effects by inhibiting apoptosis and activating neuronal survival. However, further research on a unifying hypothesis is warranted to validate our conclusions.

AUTHOR CONTRIBUTIONS

YG, ML, YW, ZL, CF, ZW, XC, JC, and HQ meet the essential authorship criteria required by the journal to be observed including (a) substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and (b) drafting the work or revising it critically for important intellectual content; and (c) final approval of the version to be published; and (d) agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

We acknowledge the Scientific and Technical Innovation Team of Zhengzhou University (No. 201310459017), the National Significant new drug creation "the twelfth-five-plan" new drug candidates (No. 2012ZX09103101-011), and the National Natural Science Foundation of China (No. 81330075) for the financial support.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 21 November 2017
doi: 10.3389/fphar.2017.00817

Antiepileptic and Neuroprotective Effects of Oleamide in Rat Striatum on Kainate-Induced Behavioral Seizure and Excitotoxic Damage via Calpain Inhibition

*Hye Yeon Nam, Eun Jung Na, Eunyoung Lee, Youngjoo Kwon and Hwa-Jung Kim**

College of Pharmacy, Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea

Oleamide was first known as a sleep-inducing fatty acid amide, and later shown to have wide range of neuropharmacological effects upon different neurochemical systems. However, the effects of oleamide on brain damage have scarcely been studied, and the molecular mechanisms and sites of its action remain elusive. Kainic acid (KA) has been used to produce an epileptic animal model that mimics human temporal lobe epilepsy and to induce calpain-activated excitotoxicity, which occurs in numerous neurodegenerative disorders. In this study, we examined whether oleamide protects against the KA-induced excitotoxic brain damage accompanied by behavioral seizure activity and neuronal cell death. Moreover, whether these effects of oleamide were mediated by calpain activity-related cellular mechanisms was investigated. KA-induced epileptic rats were produced by an intrastriatal injection of KA (5 nmole). Oral administration of oleamide (0.5, 2, and 10 mg/kg) 30 min prior to the KA injection showed dose-dependent inhibition of the KA-induced behavioral seizure activities that were monitored starting from 60 to 180 min post-surgery. Further repetitive oral administration of oleamide (once per day) for the next 4 consecutive days post-KA injection produced significant neuroprotection against the disrupted neuronal integrity that resulted from KA-induced excitotoxic damage that was also demonstrated by staining of striatal tissue sections with cresyl violet, hematoxylin/eosin, and fluoro-Jade B. In addition, oleamide blocked the KA-induced cleavage of cyclin-dependent kinase-5 coactivator (Cdk5-p35) and collapsin response mediator protein-2, which are believed to be mediated by calpain activation in striatal tissues dissected from KA-induced epileptic rats. Oleamide also reversed the KA-induced reduction in expression of an endogenous calpain inhibitory protein, calpastatin, and a marker of synaptic activity, synapsin-II. The hypothesis that oleamide could induce direct calpain inhibition was further investigated using *in vitro* calpain assays in both brain tissue and a cell-free and calpain-overexpressed neuronal cell system. These findings together suggest that oleamide has protective effects against excitotoxicity-induced neuronal death and behavioral seizure, partly via its direct calpain inhibitory activity.

Keywords: oleamide, kainic acid, epilepsy, calpain, neuroprotective effect

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Wladyslaw-Lason, Institute of Pharmacology PAS, Poland
Anna Maria Pittaluga, Università di Genova, Italy

***Correspondence:**
Hwa-Jung Kim
hjkim@ewha.ac.kr

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *01 April 2017*
Accepted: *27 October 2017*
Published: *21 November 2017*

Citation:
Nam HY, Na EJ, Lee E, Kwon Y and Kim H-J (2017) Antiepileptic and Neuroprotective Effects of Oleamide in Rat Striatum on Kainate-Induced Behavioral Seizure and Excitotoxic Damage via Calpain Inhibition. Front. Pharmacol. 8:817. doi: 10.3389/fphar.2017.00817

INTRODUCTION

Oleamide (*Cis*-9,10-octadecenoamide) is a centrally acting fatty acid amide that belongs to the family of endogenous lipid signaling molecules that includes endocannabinoids, anandamide, N-palmitoylethanolamine, and N-oleoylethanolamine (Fowler, 2004). Oleamide was first found to exist in the cerebrospinal fluid of sleep-deprived animals and act as an endogenous sleep-inducing substance (Cravatt et al., 1995). Besides inducing sleep, systemic administration of exogenous oleamide has been shown to produce a variety of central nervous system (CNS) effects (Boger et al., 2000b; Leggett et al., 2004), including elicitation of hypothermia (Fedorova et al., 2001), analgesia, memory (Murillo-Rodriguez et al., 2001; Akanmu et al., 2007), food intake (Martinez-Gonzalez et al., 2004), hypo-locomotion (Huitron-Resendiz et al., 2001), and reduction of pentylenetetrazole-induced epileptic behavior (Wu et al., 2003; Solomonia et al., 2008). Furthermore, it was recently reported that oleamide reduces amyloid-β (Aβ) accumulation via enhanced microglial phagocytosis and suppresses inflammation after amyloid Aβ deposition (Ano et al., 2015).

Although a wide range of neuropharmacological actions of oleamide have been suggested in several neurotransmitter systems, its effects on brain damage are less well studied and its mechanisms of action remain elusive. The endocannabinoid system is known to play a role in the cell death/survival decision and to improve glutamate homeostasis, thus reducing excitotoxicity (Zoppi et al., 2011). As oleamide is structurally similar to an endogenous fatty acid amide, anandamide, it has been speculated that oleamide possesses full agonist activity on the cannabinoid (CB_1) receptor (Boger et al., 2000a; Leggett et al., 2004). On the other hand, other reports have indicated that oleamide has negligible or no effects on the CB_1 receptor (Mechoulam et al., 1997; Lichtman et al., 2002). Other neuronal receptor systems have also been reported to be associated with the actions of oleamide. Oleamide has shown to inhibit gap junction (connexin)-mediated cell–cell communication (Guan et al., 1997; Boger et al., 1998), and to modulate ionotropic γ-amino butyric acid ($GABA_A$) receptors (Verdon et al., 2000), and serotonergic 5-HT_1, 5-$HT_{2A/2C}$, and 5-HT_7 receptors (Huidobro-Toro and Harris, 1996; Mueller and Driscoll, 2009). However, little is known about the neuroprotective effect of exogenous oleamide against neuronal death or its underlying intracellular mechanisms.

Kainic acid (KA) has been used to produce an epileptic animal model that mimics human temporal lobe epilepsy (Nadler, 1981) and induce sustained neuronal depolarization and hyperexcitability (Frerking et al., 1998), leading to excitotoxicity in various brain regions including striatum, hippocampus, cerebral cortex, amygdala, and nucleus accumbens, etc. (Zaczek et al., 1980; McGeer and Zhu, 1990; Ferrer et al., 1995; Araujo et al., 2008). Excitotoxicity is a major mechanism of neuronal death in acute brain injury such as stroke, epilepsy, and traumatic brain injury, and is also related to chronic neuronal degenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and others (Lau and Tymianski, 2010). As glutamate is the major excitatory neurotransmitter in the CNS, overstimulation of its receptors increases intracellular Ca^{2+} levels by directly opening post-synaptic ion channels and secondarily affecting Ca^{2+} homeostatic mechanisms (Chen et al., 2000). An increase in the cytoplasmic Ca^{2+} level activates a number of Ca^{2+}-dependent proteases, particularly calpains, leading to various types of cell death (Araujo et al., 2010) that include necrotic cell death through various different pathways, such as those involving membrane breakdown, cytoskeletal alterations, and nitric-oxide-derived free radicals (Norenberg and Rao, 2007; Mehta et al., 2013), and also triggers apoptosis (Mattson, 2007; Quintanar et al., 2012) or autophagic neuronal death (Kim et al., 2009). Therefore, interest in calpain inhibitors has been growing in an effort to overcome the calpain activity related to cell death that plays a main role in such a wide variety of CNS disorders. The high concentration and activity of calpain that accompanies neuronal degeneration has been seen in the brains of epileptic animal models (Bi et al., 1997; Feng et al., 2011), and KA-induced excitotoxic injury also appear to be prevented by calpain inhibitors (Fitzpatrick et al., 1992).

The present study investigates the antiepileptic and neuroprotective effects of oleamide through calpain inhibition as a potential intracellular mechanism. The ability of oleamide to inhibit calpain activity was demonstrated in both brain tissues of KA-induced *in vivo* epileptic rat models and *in vitro* neuronal systems.

MATERIALS AND METHODS

Chemicals and Reagents

Oleamide, KA, carbamazepine, calpeptin, cresyl violet, hematoxylin and eosin were purchased from Sigma–Aldrich (St. Louis, MO, United States). Fluoro-jade B was purchased from Histo-Chem Inc. (Jefferson, AR, United States). E64d [2S,3S-*trans*-(ethoxycarbonyloxirane-2-carbonyl)-L-leucine-(3-methyl butyl) amide] was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY, United States). The μ-calpain was purchased from Calbiochem (Darmstadt, Germany). Oleamide was suspended in 0.2% methyl cellulose.

Animals, Surgery, and Drug Administration

Sprague Dawley rats (230–240 g body weight) were purchased from Orient Bio Department (Kyungki-do, Korea). The animals were housed individually in a temperature- (20 ± 1°C) and relative humidity-controlled environment and maintained on a 12-h light/12-h dark cycle. All animal experiments were conducted according to ethical procedures and approved by the Institutional Animal Care and Use Committee of Ewha Womans University (Approval No. Ewha-IACUC 2013-01-041).

For surgery, rats were anesthetized with zoletil (20 mg/kg) and xylazine (9.5 mg/kg) and placed in a stereotaxic apparatus. A Hamilton syringe was used with a mini-pump (Nanometer Injector Syringe Pump; Harvard Apparatus, Holliston, MA, United States) to inject the rats with KA (5 nmole, 0.5 μl)

or vehicle (saline, 0.5 μl) intrastriataly at coordinates of 1.2 mm posterior, ± 2.5 mm lateral, and ± 5.5 mm ventral, relative to the bregma. Rats were sacrificed 5 days after surgery. Oleamide (0.5, 2, and 10 mg/kg, p.o.) or vehicle (0.2% methyl cellulose) was orally administered 30 min before the surgery, and administered daily for 4 days after the surgery.

Monitoring Behavioral Seizures Induced by Intrastriatal Injection of KA

Rats were continuously observed throughout the 3-h period by observers blinded to the treatment. Behavioral seizures were monitored and scored starting from 60 min through 180 min post-surgery. The seizure counts and score were recorded every 10 min to produce representative counts of seizure expression during that period. The convulsive behavior scale consisted of the following seven stages: stage 0, normal behavior; stage 1, wet dog shakes and mouth or facial movements; stage 2, head nodding; stage 3, forelimb clonus; stage 4, rearing; stage 5, rearing and falling; stage 6, tonic seizure or death (Racine, 1972; Sperk et al., 1985).

Sample Preparation and Western Blotting

Striatal tissues were collected in cold lysis buffer containing 1% Triton X-100, 1 mM EDTA in phosphate-buffered saline (PBS), protease inhibitor cocktail, homogenized, and centrifuged at 10,000 × *g* for 10 min at 4°C. Protein concentration was determined using a BCATM protein assay kit (Thermo Fisher Scientific, Waltham, MA, United States) and assessed by Western blotting. Equal aliquots of the samples were denatured at 100°C, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Billerica, MA, United States). Membranes were incubated in a blocking buffer containing 5% BSA in TTBS for 1-h at room temperature. Immunodetection was performed by incubating membrane blots overnight at 4°C separately with the following primary antibodies (1:1000): anti-CRMP-2 (IBL, Gunma, TS, Japan), anti-Cdk5-p35/25, anti-calpastatin, and anti-synapsin-II (Cell Signaling, Dallas, TX, United States). For chemiluminescent detection, membrane blots were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) for 2-h at room temperature. Data collection and processing of the integrated optical density of the bands were performed with a luminescent image analyzer (LAS-3000) and IMAGE GAUSE software (Fuji Photo Film, Japan).

Histological Analysis

Cresyl violet and hematoxylin/eosin (H&E) staining were used to stain tissue sections for histological examination and measurement of neuronal loss. Rats ($n = 3$ per group) were anesthetized with zoletil (20 mg/kg) and xylazine (9.5 mg/kg) and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Perfused brains were post-fixed in 4% paraformaldehyde in PBS overnight and subjected to increasing concentrations of alcohol overnight. The brain tissue blocks were embedded in paraffin and the paraffin blocks were cut into a series of 5-μm-thick slices and stained with 0.1% cresyl violet and H&E. All sections were coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, United States) and were examined with a light microscope (Carl Zeiss, Gottingen, Germany), and photographs were taken with an AxioCam HRC digital camera (Carl Zeiss, Gottingen, Germany).

Fluoro-Jade B (FJB) staining was used to identify degenerating neurons. Briefly, the slides were immersed in 100% ethanol for 3 min, followed by 70% ethanol for 2 min and distilled water for 2 min. The slides were then transferred to 0.06% potassium permanganate for 15 min and gently agitated. After rinsing in distilled water for 2 min, the slides were incubated for 30 min in 0.001% FJB, which was freshly prepared by adding 20 ml of a 0.01% stock FJB solution to 180 ml of 0.1% acetic acid with gentle shaking in the dark. After rinsing three times for 1 min in distilled water, the slides were dried, dehydrated in xylene, and coverslipped.

In Vitro μ-Calpain Assay

There are two prototypical calpain forms, m-calpain, and μ-calpain. The m-calpain, composed of the catalytic subunit calpain II, is located at the membrane and requires 0.2–0.8 mM concentrations of Ca^{2+} for activation. In contrast, μ-calpain, composed of the catalytic subunit calpain II, is located in the cytosol or near the membrane and is activated by 2–80 μM concentrations of Ca^{2+} *in vitro*. Therefore, μ-calpain activity can be affected by minute changes Ca^{2+} concentration. The direct inhibitory effect of oleamide was examined by analyzing μ-calpain activity in calpain 1 (CAPN1)-overexpressing neuronal cells (Lee et al., 2013). In order to measure the μ-calpain inhibitory activity of compounds in SH-SY5Y human neuroblastoma cells, the human CAPN1 gene, which encodes the μ-calpain catalytic subunit, was synthesized (Camins et al., 2006). SH-SY5Y human neuroblastoma cells (ATCC CRL-2266) were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained at 37°C in humidified conditions under 5% CO_2. The medium was changed twice weekly, and cultures were split in a 1:5 ratio weekly. For experiments, SH-SY5Y cells were plated in 6-well plates for 48 h. The medium was then removed and replaced with fresh medium without serum, and the cells were maintained for the time periods indicated.

SH-SY5Y cells were transiently transfected with 3 μg of CAPN1-encoding pcDNA3.1/His A expressing the catalytic domain of μ-calpain. After transfection, cells were incubated with each compound in reaction buffer. The cleavage product of pep1 by μ-calpain was measured with the microplate fluorescence reader (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA, United States) in kinetic mode at 37°C for 210 min. Fluorescence was measured at 320 nm (ex)/420 nm (em).

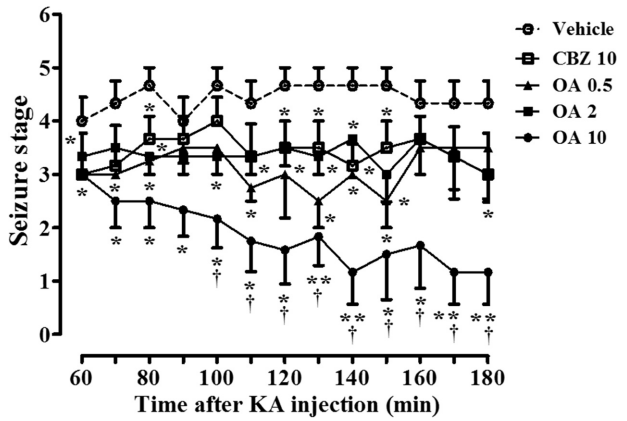

FIGURE 1 | Oleamide (OA) reduces KA-induced behavioral seizure severity throughout the 3-h rating period, and is comparable to carbamazepine (CBZ). Seizures were initiated in rats by intrastriatal injection of KA (5 nmol) into the right striatum (the left striatum was treated with saline). Rats were orally administered either vehicle (0.2% methyl cellulose), OA (0.5, 2, and 10 mg/kg), or CBZ (10 mg/kg) 30 min before the KA injection. Behavioral seizures were monitored by blinded raters for 3-h after the surgery and scored for 2-h from 60 to 180 min. The data are the means ± SEM of 4–6 independent experiments. $^{*}p < 0.01$ and $^{**}p < 0.001$ (ANOVA) compared with KA-only data (vehicle). $^{\dagger}p < 0.05$ vs. CBZ-treated data.

Calpain Substrate Cleavage Assay in *in Vitro* μ-Calpain-Treated Striatal Tissue Extracts

Normal rat striatal tissue lysates (20 μg) were incubated with μ-calpain (0.0125 U/ug) in the presence of 1 mM $CaCl_2$ for 30 min at 37°C. Independently, oleamide (30, 100 μM) was co-incubated in the same conditions to identify that the cleavage of collapsin response mediator protein-2 (CRMP-2) and cyclin-dependent kinase-5 (Cdk5)-p35 were mediated by calpain. Western blotting was performed for detection of CRMP-2 and Cdk5-p35.

Statistical Analysis

Statistical analyses were performed with the Newman–Keuls test and analyses of variance (one-way ANOVA) with GraphPad Prism version 5.0d (GraphPad Software, Inc., La Jolla, San Diego, CA, United States). The p-value < 0.05 was considered statistically significant. All results were expressed as the mean ± SEM of at least three independent experiments.

RESULTS

Kainic Acid (KA)-Induced Behavioral Seizure Activity and Antagonizing Effect of Oleamide

Systemic (intraperitoneal) administration of KA in rodents induced epileptic seizures similar to human temporal lobe epilepsy, with spontaneous seizures, as well as seizure-induced neuronal cell death (Ben-Ari, 1985; Bortolatto et al., 2011). A clinically used prototype antiepileptic drug, carbamazepine, has shown to produce the dose-dependent protective effects on spontaneous seizures in rats with KA-induced epilepsy by intraperitoneal injection at concentration ranges of 30–100 mg/kg (Grabenstatter et al., 2007), and the protection against pentylenetetrazole-induced seizure by oral administration at 10–20 mg/kg (Reeta et al., 2011). We first examined whether oleamide inhibits KA-induced epileptic behavior. Rats were pretreated with an oral injection of oleamide (0.5, 2, or 10 mg/kg suspended in 0.2% methylcellulose or vehicle (0.2% methylcellulose) 30 min prior to intrastriatal infusion of KA (5 nmole/0.5 μl). **Figure 1** shows the time course of behavioral seizures in each treatment group. In the vehicle group, rats showed extreme seizures behaviors. Maximum average Racine's score reached 4.4 points (Racine, 1972). Oleamide (0.5, 2, 10 mg/kg) produced anticonvulsive effects on KA-induced behavioral seizures in a dose-dependent manner. Especially, oleamide at 10 mg/kg dramatically reduced the seizure scores (from 4.3 to 1.2 at 180 min) that were significantly greater than that of same concentration (10 mg/kg) of carbamazepine (3.0 at the same time point).

Protective Effects of Oleamide against KA-Induced Neuronal Damage in Striatum

It has been shown that KA induces internucleosomal DNA fragmentation and loss of striatal neurons (Wang et al., 2008). We examined whether oleamide is protective against KA-induced excitotoxic neuronal damage, using histological staining analyses. Cresyl violet and hematoxylin/eosin (H&E) staining data clearly showed the KA-induced neuronal damage instriatal brain tissue sections, which is similar to other reports that KA administration resulted in the presence of mainly pyknotic nuclei (Nash et al., 1991; Liao et al., 2016).

H&E staining showed that striatal tissues from rats exposed to KA (5 nmole) exhibited extensive cell loss and pyknotic nuclei at the neuropil. In contrast, rats pretreated with oleamide prior to KA injection showed a significant reduction in pyknotic nuclei, while pyknotic nuclei remained in cells of vehicle-treated KA-epileptic rat striatal tissues. In addition, the protective effect of oleamide was also observed in cresyl violet staining, which enables visualization of neuronal damage and cell dispersion (**Figure 2A**). The number of damaged neuronal cells was measured by counting cells in each of two fields randomly selected from each of three separate striatal tissue sections of three rats per group. The general criteria used to score damaged cells included the number of hyperchromatic nuclei and cytoplasmic vacuolation. Quantitative analyses (**Figures 2B,C**) revealed a dramatic protective effect of oleamide (10 mg/kg) following KA administration in the striatum, as assessed by both cresyl violet staining ($p < 0.001$) and H&E staining ($p < 0.001$), as compared with KA alone.

The protective effects of oleamide against neuronal damage were further confirmed using staining with FJB, which specifically stains dying neurons and becomes fluorescent green. FJB is an anionic fluoresce in derivative useful for histological staining

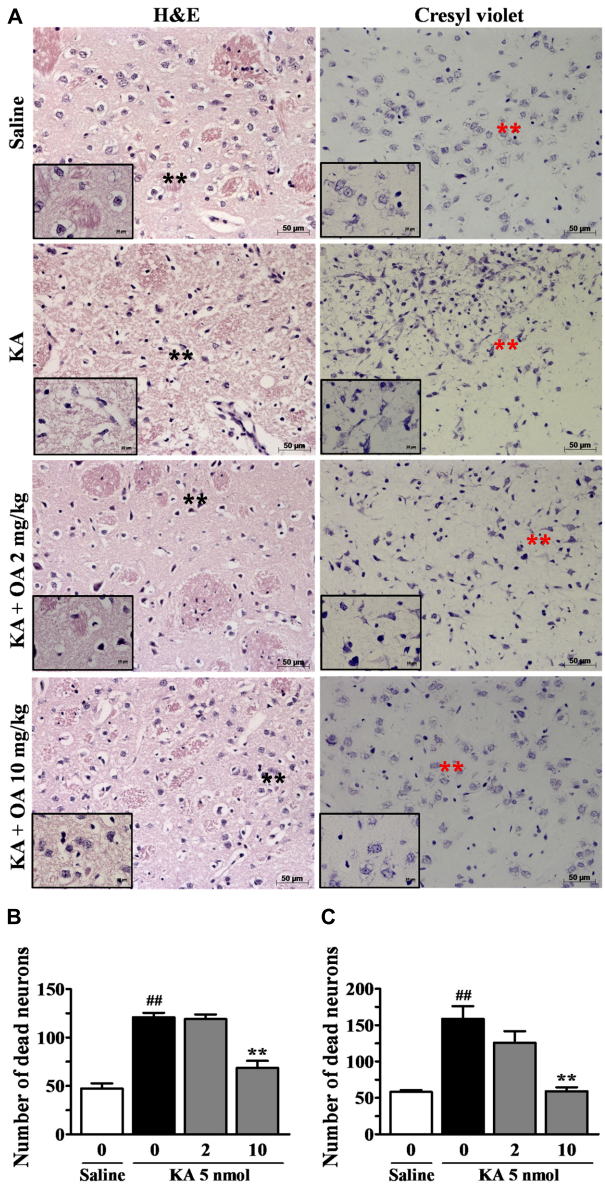

FIGURE 2 | Protective effects of oleamide (OA) on KA-induced neuronal damage. Brains were coronally sectioned into 5 μm sections 5 days after the stereotaxic injection of saline and KA 5 nmol. The coronal sections were mounted on slides and stained with H&E and cresyl violet **(A)**. Asterisks indicate the tissue areas that are magnified in the smaller panels. Striatal regions were enlarged ×200 and ×630. Scale bars = 50 and 10 μm. **(B,C)** The number of damaged neuronal cells per brain (n = 3) section was quantitated using the Image J program. ##$p < 0.001$ vs. saline, and **$p < 0.001$ vs. vehicle.

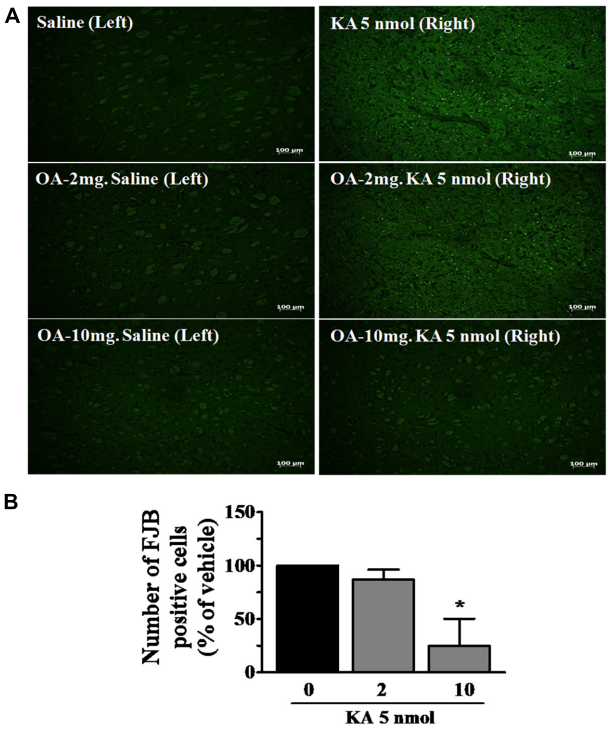

FIGURE 3 | Neuroprotective effects of oleamide (OA) against KA-induced neuronal cell death. Fluoro-jade B (FJB) staining was performed on coronally sectioned rat brains to identify damaged neurons 5 days after the induction of KA. **(A)** Representative magnification (100×) photomicrographs showing FJB-positive neurons indicating neurodegeneration in the striatum. Three animals were randomly assigned to each condition. Scale bar = 100 μm. **(B)** FJB-positive neurons were quantitated using the Image J program. *$p < 0.05$ vs. vehicle.

of neurons undergoing cellular degeneration (Schmued and Hopkins, 2000; Miyamoto et al., 2008; Furtado et al., 2011). Numerous FJB-positive cells were observed in the striatum following KA (5 nmole) injection, as similarly seen in the hippocampus by other report (Miyamoto et al., 2008). In the oleamide-treated animals, only a few positive cells were found in the striatum, and the effect was dose-dependent (**Figure 3A**). No FJB-positive cells were detected in the left striatum of sham-operated and oleamide-treated control rat brains. Quantification of FJB-stained cells in the region revealed a statistically significant decrease in the number of stained cells following oleamide (10 mg/kg) administration (**Figure 3B**). FJB staining data provide further evidence confirming the protective effects of oleamide against KA-induced excitotoxic brain damage.

Inhibitory Effect of Oleamide on KA-Induced Calpain Activation in Epileptic Rat Striatal Tissue

It was shown that injection of KA into the striatum increased calpain mRNA (Campbell et al., 2004). Calpain enzyme has been shown to play a central role in KA-induced excitotoxicity by cleaving a large number of substrates, including a Cdk5 coactivator (p35) and CRMPs (Bevers and Neumar, 2008). KA-induced calpain-specific Cdk5-p35/25 pathway activation, represented by the conversion of Cdk5-p35 to p25, was also blocked by pretreatment with oleamide (**Figure 4A**). It was previously shown that CRMP-2 (∼62 kDa) is specifically cleaved into a 58 kDa protein under ischemic conditions and that CRMP-2 cleavage is mediated by calpain (Chung et al., 2005). The

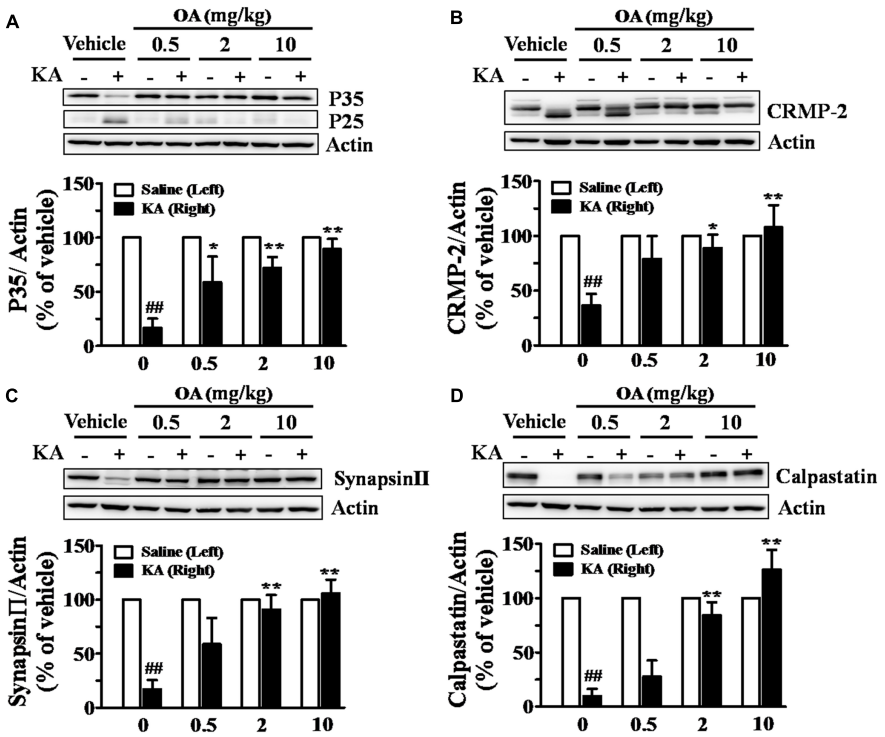

FIGURE 4 | Inhibitory effects of oleamide (OA) on KA-induced calpain activation in rat striatum. Rats were administered OA (0.5, 2, and 10 mg/kg, p.o.) or vehicle (0.2% methyl cellulose) 30 min before intrastriatal injection of KA (5 nmol), whereas the left striatum was treated with saline only, and given repeated oral doses of OA daily for the next 4 days. Striatal lysates were subjected to Western blot analysis using anti-Cdk5-p35 **(A)**, anti-CRMP-2 **(B)**, anti-synapsin-II **(C)**, and anti-calpastatin **(D)**. Data are the means ± SEM of 3–7 experiments. $^{\#\#}p < 0.001$ vs. vehicle, $^{*}p < 0.01$ and $^{**}p < 0.001$ vs. KA-treated vehicle.

oleamide-induced change in cleavage patterns of those substrates was examined in striatal tissues of KA-induced epileptic rats. Oral administration of oleamide (0.5–10 mg/kg) antagonized KA-induced cleavage of CRMP-2, as reflected by a dose-dependent increase in the level of uncleaved 62 kDa CRMP-2 and a decrease in the concentration of 58 kDa CRMP-2 (**Figure 4B**). Synapsin-II protein is a marker of synaptic activity and plasticity (Zurmohle et al., 1996; Ferreira et al., 1998) and its level has been shown to be reduced in epileptic or neuronal death conditions (Karanian et al., 2007). We observed that the level of synapsin-II was markedly decreased in KA-induced epileptic rat striatal tissues, and that oleamide prevented the KA-induced reduction of synapsin-II to control levels in the normal rat striatum (**Figure 4C**). Our data suggest a protective effect of oleamide against KA-induced disruptions in synaptic integrity. In addition, oleamide protected the expression of an endogenous calpain inhibitory protein, calpastatin, the level of which was reduced by KA (**Figure 4D**).

Calpain Inhibitory Effects of Oleamide in *in Vitro* μ-Calpain-Treated Striatal Tissue Extracts and in Calpain 1 (CAPN1)-Overexpressed Neuronal Cells

The cell-based μ-calpain assay was performed to investigate the μ-calpain inhibitory activity of oleamide in SH-SY5Y cells. μ-Calpain is endogenously expressed in SH-SY5Y cells, but its activity is not potent enough to sufficiently cleave the exogenous substrate pep1. Therefore, the large subunit of μ-calpain, CAPN1, was additionally transfected to visibly measure the compound μ-calpain inhibitory activity in SH-SY5Y cells. E64D, an irreversible calpain inhibitor, was prepared by esterification of the free carboxylic acid group of E64C to improve cell permeability. The results showed that μ-calpain activity was highest in CAPN1-transfected cells. Treatment with oleamide (30, 100 μM) reduced μ-calpain activity with better potency than did E64D (100 μM). Oleamide (30 μM) produced an inhibitory effect to a similar degree as calpeptin (30 μM), and the higher concentration of oleamide (100 μM) significantly decreased the enhanced μ-calpain activity to the level of control cells (**Figure 5**). This result confirms the direct calpain inhibitory effect of oleamide, suggesting the possibility that it can be used as a novel calpain inhibitor.

The *in vitro* calpain-inhibitory effect of oleamide was further confirmed by adding oleamide to rat striatal tissue extracts incubated with purified μ-calpain. As shown in **Figure 6**, oleamide (30, 100 μM) remarkably decreased the calpain-induced cleavage of both CRMP-2 and Cdk5-p35. These results suggest the possibility that oleamide is a novel calpain inhibitor.

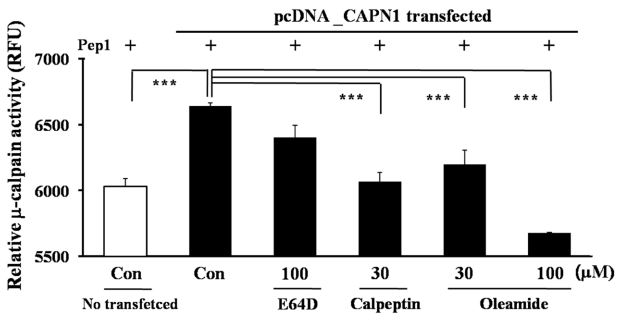

FIGURE 5 | Inhibition of calpain activity by oleamide (OA) in μ-calpain overexpressed SH-SY5Y cells. μ-Calpain (CAPN1)-overexpressed SH-SY5Y cells were incubated with substrate pep 1 (peptide derived from the Cdk5-p35 cleavage site) and compounds. The relative change in fluorescence induced by μ-calpain-mediated pep1 cleavage was quantified. Fluorescence was measured at 320 nm (ex) and 420 nm (em). ***$p < 0.001$ vs. μ-calpain-transfected control.

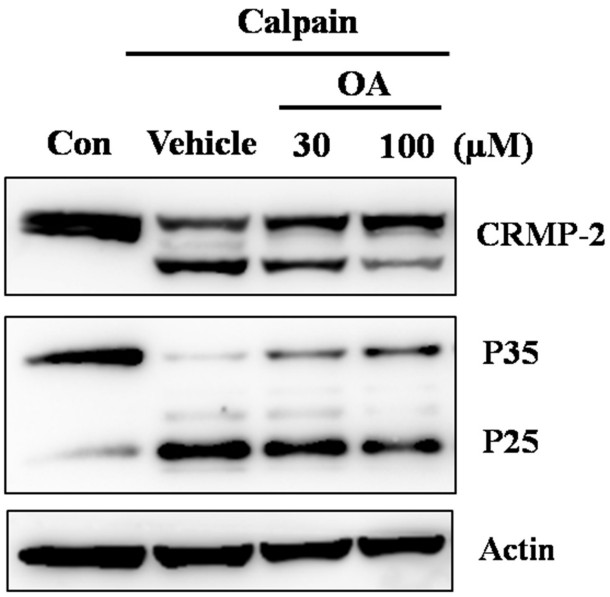

FIGURE 6 | Inhibition of calpain activity by oleamide in rat striatum tissue lysates. Rat striatum tissue lysates (20 μg) were incubated with μ-calpain (0.0125 U/ug) in the presence of 1 mM $CaCl_2$ for 30 min at 37°C. OA (30, 100 μM) was co-incubated in the same conditions to assess whether the cleavage of CRMP-2 and Cdk5-p35 were mediated by calpain. Western blotting was performed to detect cleavage of CRMP-2 and Cdk5-p35.

DISCUSSION

The present study investigates the *in vivo* antiepileptic and neuroprotective effects of exogenous oleamide against KA-induced excitotoxic brain damage, and asks whether calpain inhibition is an intracellular mechanism of those effects. We demonstrated the preventive effects of oleamide against epileptic behavior in KA-induced epileptic rat model and against excitotoxicity-induced calpain activation that leads to neuronal death. The ability of oleamide to inhibit calpain activity was examined in both brain tissue of a KA-induced epileptic rat model and cultured neurons.

Oleamide is an endogenous fatty acid amide and shares a structure and some characteristics with the endocannabinoid anandamide. Both oleamide and anandamide are degraded by fatty acid amide hydrolase (Boger et al., 2000a). While many biological effects of oleamide are documented, its molecular and signaling mechanisms are less well defined than those of cannabinoids. The endocannabinoid system has been identified as a potential target for the treatment of several disorders of the CNS, including epilepsy and excitotoxicity (Marsicano et al., 2003; Monory et al., 2006). An increasing number of studies have implicated the endogenous fatty acid amide system as a new target for neuronal damage, mainly studies on anandamide and fatty acid amide hydrolase inhibitors (Karanian et al., 2007; Naidoo et al., 2012). Several studies have shown that endocannabinoid deficiency may contribute to the pathophysiology of chronic pain including migraine and inflammatory pain (Lastres-Becker et al., 2002; Clapper et al., 2010; Nozaki et al., 2015). The novel fatty acid amide hydrolase inhibitor, URB-597, has been demonstrated to produce anticonvulsant effect on seizures induced by pentylenetetrazole in rats (Vilela et al., 2013), and to block neuronal hyperactivity in neurons (Nozaki et al., 2015), which can be considered a similar mechanism to that seen in epilepsy. Interestingly, the administration of an endocannabinoid uptake inhibitor (AM404) that kept the fatty acid amide levels high to the rats injected intrastriatally with 3-nitropropionic acid, a toxin that selectively damages striatal GABAergic neurons, was shown to attenuate hyperactive motor disturbances (Lastres-Becker et al., 2002). These reports suggest that fatty acid amides can play important roles as signaling molecules that are involved in mechanisms for epilepsy and motor hyperactivity. Another fatty acid amide hydrolase inhibitor (AM374) that was reported to cause a prolonged elevation of the anandamide level in the brain reduced KA-induced seizures by promoting CB_1 receptor signaling (Karanian et al., 2007; Shubina et al., 2015). Thus, fatty acid amide hydrolase inhibitors have been suggested to be relevant to the excitotoxic protection by enhancement of the endocannabinoid system, which is elicited via inhibition of endocannabinoid degradation.

Although oleamide is an endogenous fatty acid amide, which is degraded by fatty acid amide hydrolase, only a few of studies have reported on the *in vivo* antiepileptic and neuroprotective effects by exogenously administered oleamide. We first demonstrated that oral administration of oleamide significantly reduced seizure behavior in KA-induced epileptic rats. The KA-induced chronic epileptic animal model has been known to mimic human temporal lobe epilepsy and status epilepticus (Nadler, 1981). Many reports have shown that changes in the hippocampus and/or cortex are mainly involved in epileptic seizures. The striatum, however, is also well reported as an important brain region of the neuronal damage with relation to the occurrence of convulsions, including KA-induced seizures in rats (Pisa et al., 1980). In rats induced epilepsy by injection of KA into the striatum, anti-seizure effect of

oleamide was more potent than that of carbamazepine, a clinically long-used anticonvulsant agent (**Figure 1**). Previously, the anticonvulsant effect of oleamide was only shown to produce against the behavioral seizures induced by pentylenetetrazole, the most commonly used acute seizure model (Wu et al., 2003; Solomonia et al., 2008). According to the Wu et al.'s (2003) report, intraperitoneal administration of oleamide (43.8–700 mg/kg) to mice significantly attenuated the latency of seizure onset induced by pentylenetetrazole (85 mg/kg, i.p.) that was administered 30 min after the injection of oleamide, but produced no effect on seizures induced by other convulsive agents, such as picrotoxin, semicarbazide, strychnine, and caffeine. This report does not fully explain the selective action of oleamide on pentylenetetrazole-induced seizures, and the *in vivo* dose range of oleamide used in the report is exceptionally higher than that reported by other researchers. In another short report, oleamide in a dose of 10 mg/kg injected (i.p.) to rats was shown to produce anti-seizure effect specifically on the degree (severity) of convulsions, but not decrease the duration and latency of convulsions (Solomonia et al., 2008).

In addition, we found that oleamide prevents KA-induced neuronal death through histological analyses of cellular integrity in striatal sections of KA-induced epileptic rats (**Figures 2**, **3**). The striatum has been demonstrated to express a large number of binding sites for all classes of glutamate receptors (Albin et al., 1992; Wullner et al., 1994; Miyamoto et al., 2008), and the intrastriatal injection of KA has been to lead to substantial neuronal loss in striatal tissues (Wang et al., 2006). Apoptotic and necrotic death of neurons is involved in KA-induced excitotoxicity *in vivo* (Wang et al., 2005; Vincent and Mulle, 2009). The KA-induced striatal damage observed in our demonstrated experimental condition can be considered from direct toxic effect of KA, presumably through excessive stimulation of striatal glutamate receptors, since the neuronal damage was observed in only right side of striatum where KA was directly injected, but not in saline-injected left side of striatum. Excessive stimulation of glutamate receptors and neuronal damage due to KA is thought to be a result of a large influx of Ca^{2+} into neurons and by a dysfunction of downstream signaling systems (Koh et al., 1990; Lipton and Rosenberg, 1994) including calpain activation.

It has been reported that neuronal damage can spread from the KA-injected striatum into contiguous structures (Zaczek et al., 1980), and can induce locomotor changes and epileptogenesis (McGeer and Zhu, 1990). Although KA-induced seizures are followed by extensive neurodegenerative changes, the seizure-induced brain damage is still debated (not conclusively demonstrated) both in humans and in animal models of epilepsy (Rossini et al., 2017).

Alterations in Ca^{2+} homeostasis lead to persistent, pathologic overactivation of calpain in a number of neurodegenerative diseases (Vosler et al., 2008). Initially, calpain activation was thought to cause only necrotic cell death, while activation of caspase led to programmed cell death. There is a shared mechanism of calpain activation in neurodegenerative diseases, such as epilepsy, Alzheimer's disease, and Parkinson's disease (Lau and Tymianski, 2010; Lopatniuk and Witkowski, 2011). In particular, calpain-specific cleavage of Cdk5-p35 to p25 has been implicated in the neurological damage seen in many neurological disorders (Kanungo et al., 2009; Cheung and Ip, 2012). Calpains have long been implicated in neuronal cell death induced by triggers of neuronal injury including excitotoxicity. Putkonen et al.'s (2011) reported that KA produced a dose-dependent increase in intracellular Ca^{2+}concentration and raised calpain activity, followed by induction of phosphorylation of Cdk5 and cleavage of Cdk-p35, which are believed to be involved in KA-mediated degeneration of glutamatergic synapses in the rat hippocampus (Putkonen et al., 2011). Also in the striatum, it has been shown that intrastriatal injection of KA increases calpain mRNA (Campbell et al., 2004). In addition, calpain activation is involved in manganese-induced neuronal death in rat striatum (Quintanar et al., 2012).

Since many calpain inhibitors have been shown to produce antiepileptic effects (Lubisch et al., 2000), we postulated that oleamide can also affect calpain activity, and investigated cleavage of the representative calpain substrate protein, Cdk-p35 in KA-induced epileptic rat striatal tissues. We found that the KA-induced enhancement of Cdk5-p35 cleavage to p25 was significantly blocked by oral administration of oleamide (**Figure 4A**), implying that oleamide could produce antiepileptic and neuroprotective effects by the mechanism related to calpain inhibition.

The KA-induced significant neuronal loss can lead to the axonal degeneration that is preceded by disruption of Ca^{2+} homeostasis, causing calpain activation and the proteolytic degradation of axonal proteins (Saatman et al., 1996). CRMP-2 is known to be involved in neuronal differentiation and control of neuronal polarity and axonal outgrowth (Yoshimura et al., 2006). In addition, more recent studies have reported that CRMP-2 can bind to the voltage-gated calcium channel Cav2.2, and this interaction may play a crucial role in neurotransmitter release from the presynaptic terminals of hippocampal neurons (Wang et al., 2010). In our previous study, CRMP-2 protein was shown to be altered into a cleaved form with a size of 58 kDa in the brain under ischemic conditions (Chung et al., 2005), which was later shown to be induced by calpain activation (Hou et al., 2009). Since then, it has been discovered that CRMPs are cleaved in severe neurodegenerative conditions and their expression levels are changed in several neuronal diseases. CRMP-2 was reduced in patients with temporal lobe epilepsy (Czech et al., 2004). A relatively new antiepileptic drug, lacosamide, was reported to modulate CRMP-2 and inactivate voltage-gated sodium channels, which were identified as its antiepileptic mechanisms (Saussele, 2008; Patyar and Medhi, 2010). In the present study, calpain-induced CRMP-2 cleavage was observed in KA-induced epileptic rat striatal extracts, and, similar to Cdk5-p35, oleamide significantly blocked CRMP-2 cleavage (**Figure 4B**). Additional evidence implying that oleamide can improve KA-induced synaptic dysfunction was provided by measuring the changes in the level of synapsin-II, one of the synaptic marker proteins. KA dramatically reduced the level of synapsin-II, and oleamide dose-dependently protected the synapsin-II levels (**Figure 4C**). These data indicate that oleamide can block the calpain-mediated

cleavage of substrate proteins that play important roles in the regulation of neuronal survival/death and neuronal activity.

We further confirmed the calpain inhibitory effects of oleamide by examining changes in endogenous calpastatin levels. An endogenous calpain inhibitory protein, calpastatin, has been proposed to play pro-survival roles in adult neurons under degenerative conditions, including in models of ischemia–excitotoxicity (Yang et al., 2013). Recently, exogenous transgenic expression of calpastatin was used to provide definitive evidence for calpain's involvement in sciatic and optic nerve degeneration after sectioning *in vivo* (Ma et al., 2013). Data (**Figure 4D**) showing the ability of oleamide to enhance the level of endogenous calpastatin, which is reduced in KA-induced epileptic rat brain, suggest that its antiepileptic and possible neuroprotective effects are due to calpain inhibition.

So far, oleamide was assumed to exert its various biological actions through as-yet undefined membrane receptors. The calpain inhibitory effect of oleamide has not been reported previously. We hypothesized that oleamide might play a role as a calpain inhibitor. To investigate this, *in vitro* experiments were performed. In calpain-transfected cells, oleamide was observed to decrease the μ-calpain activity more effectively than the known calpain inhibitors E64d and calpeptin (**Figure 5**). In addition, the CRMP-2 and Cdk5 degradation observed in rat brain tissue lysates directly incubated with μ-calpain were inhibited by exogenous treatment with oleamide (**Figure 6**). These results provide evidence for the possibility of direct calpain inhibition by oleamide. It can be speculated that oleamide may enter into the intracellular space through an endogenous fatty acid amide membrane transporter that has been shown to transport anandamide (Mechoulam and Deutsch, 2005), and then inhibit calpain directly and/or indirectly, however, evidence for this theory is still lacking.

Taken together, our findings reveal that exogenous oleamide attenuates not only KA-induced behavioral seizures but also calpain-mediated neuronal cell death in the brain, suggesting that oleamide is a novel promising drug candidate for various neuronal diseases including epilepsy. In addition, the antiepileptic and neuroprotective effects of oleamide could be mediated through a novel mechanism by calpain inhibition, although its direct calpain inhibitory effect needs to be further pursued.

AUTHOR CONTRIBUTIONS

HYN performed experiments, analyzed the data, and wrote the first draft of manuscript. EJN, EL, and YK analyzed data and involved in the manuscript preparation. H-JK designed and supervised the research, analyzed data, and wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), and funded by the Ministry of Education (2011-0013387).

ACKNOWLEDGMENT

We acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 19 December 2017
doi: 10.3389/fphar.2017.00921

Dual Influence of Endocannabinoids on Long-Term Potentiation of Synaptic Transmission

*Armando Silva-Cruz[1,2], Mattias Carlström[3], Joaquim A. Ribeiro[1,2] and Ana M. Sebastião[1,2]**

[1] Instituto de Farmacologia e Neurociências, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal, [2] Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal, [3] Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

Cannabinoid receptor 1 (CB_1R) is widely distributed in the central nervous system, in excitatory and inhibitory neurons, and in astrocytes. CB_1R agonists impair cognition and prevent long-term potentiation (LTP) of synaptic transmission, but the influence of endogenously formed cannabinoids (eCBs) on hippocampal LTP remains ambiguous. Based on the knowledge that eCBs are released upon high frequency neuronal firing, we hypothesized that the influence of eCBs upon LTP could change according to the paradigm of LTP induction. We thus tested the influence of eCBs on hippocampal LTP using two θ-burst protocols that induce either a weak or a strong LTP. LTP induced by a weak-θ-burst protocol is facilitated while preventing the endogenous activation of CB_1Rs. In contrast, the same procedures lead to inhibition of LTP induced by the strong-θ-burst protocol, suggestive of a facilitatory action of eCBs upon strong LTP. Accordingly, an inhibitor of the metabolism of the predominant eCB in the hippocampus, 2-arachidonoyl-glycerol (2-AG), facilitates strong LTP. The facilitatory action of endogenous CB_1R activation does not require the activity of inhibitory A1 adenosine receptors, is not affected by inhibition of astrocytic metabolism, but involves inhibitory GABAergic transmission. The continuous activation of CB_1Rs via exogenous cannabinoids, or by drugs known to prevent metabolism of the non-prevalent hippocampal eCB, anandamide, inhibited LTP. We conclude that endogenous activation of CB_1Rs by physiologically formed eCBs exerts a fine-tune homeostatic control of LTP in the hippocampus, acting as a high-pass filter, therefore likely reducing the signal-to-noise ratio of synaptic strengthening.

Keywords: endocannabinoids, cannabinoid CB_1 receptor, long-term potentiation, adenosine A_1 receptor, hippocampus

OPEN ACCESS

Edited by:
Alfredo Meneses, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Mexico

Reviewed by:
Jose M. Trigo, Centre for Addiction and Mental Health, Canada
Regina A. Mangieri, University of Texas at Austin, United States

***Correspondence:**
Ana M. Sebastião
anaseb@medicina.ulisboa.pt

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *02 August 2017*
Accepted: *05 December 2017*
Published: *19 December 2017*

Citation:
Silva-Cruz A, Carlström M, Ribeiro JA and Sebastião AM (2017) Dual Influence of Endocannabinoids on Long-Term Potentiation of Synaptic Transmission. Front. Pharmacol. 8:921. doi: 10.3389/fphar.2017.00921

INTRODUCTION

The influence of marijuana upon human cognition mostly results from its ability to interfere with the action of endocannabinoids (eCBs) in the brain. eCBs are widely recognized as fine-tune modulators of synaptic activity, their action mainly resulting from activation of G protein-coupled cannabinoid receptor type 1 receptors (CB_1R), which are widely distributed in the central nervous system, in particular in the hippocampus, cortex, basal ganglia, and cerebellum

(Herkenham et al., 1991; Matsuda et al., 1993; Tsou et al., 1998; Marsicano and Lutz, 1999; Wilson and Nicoll, 2002). CB_1Rs are localized in neurons, both excitatory and inhibitory (Katona et al., 2001; Wilson et al., 2001; Kawamura, 2006; Hoffman et al., 2010), and also in astrocytes (Navarrete and Araque, 2008). CB_1Rs are endogenously activated by eCBs, mainly the fatty acid derivatives 2-arachidonoyl-sn-glycerol (2-AG) and anandamide. eCB synthesis mostly results from cleavage of postsynaptic membrane lipids as a consequence of the activation of postsynaptic G-coupled glutamate metabotropic receptors, which are predominantly activated as a consequence of high rate of neuronal firing (Chevaleyre et al., 2006; Katona et al., 2006). eCBs thus travel in a retrograde manner to activate astrocytic and nerve-terminal located CB_1R, resulting in inhibition of neurotransmitter release, and giving rise to several forms of short-term synaptic plasticity (Freund et al., 2003; Chevaleyre et al., 2006; Kano et al., 2009; Ohno-Shosaku et al., 2012). While the inhibitory action of eCBs upon neurotransmitter release is quite consistent, their action upon synaptic plasticity induced by brief high frequency neuronal firing, as long-term potentiation (LTP), is much more controversial. Indeed, and considering only the hippocampus, a brain area important for memory encoding and the mostly used to study synaptic plasticity phenomena, there are reports showing that eCBs restrict LTP (Bohme et al., 1999; Slanina et al., 2005) while others show that they facilitate LTP (Carlson et al., 2002; De Oliveira Alvares et al., 2006). This is intriguing since LTP is a compelling cellular model for learning and memory (see Nicoll, 2017), and exogenous cannabinoids, including the phytocannabinoids present in marijuana and the synthetic CB_1Rs agonists, have a negative impact upon learning and memory in humans and in laboratory animals (Miller et al., 1977; Lane et al., 2005; Sousa et al., 2011; Mouro et al., 2017). Elegant studies aiming at understanding the influence of eCBs upon LTP in different cell types or circuits in the hippocampus show that the action of eCBs may vary according to the cell type where the CB_1Rs sit (Monory et al., 2015) as well as the hippocampal circuit where LTP is induced (Wang et al., 2016). Knowing that eCBs are formed as a function of neuronal activity, we hypothesized that the influence of eCBs upon LTP could also vary as a function of the pattern of neuronal firing that induces plasticity. Evidence for that would not only contribute to further clarify reasons for discrepant data in the literature but also to better insight on the subtleties eCBs use to control synaptic strengthening. The present work was thus designed to evaluate the influence of eCBs upon hippocampal LTP induced by two types of stimulation, while keeping a θ-burst stimulation pattern, known to be related to hippocampal-dependent memory function (Buzsáki, 2002). We used a weak or a strong-θ-burst train of stimulation since previous evidence lead us to hypothesize that modulation of strong or weak forms of LTP may differ. Data obtained allow to suggest that eCBs act as a high pass filter, inhibiting LTP of low magnitude while facilitating robust LTP. Thus, eCBs likely reduce the signal-to-noise ratio of activity-dependent synaptic strengthening at the CA1 area of the hippocampus.

MATERIALS AND METHODS

Animals

The experimental protocols were approved by Institutional Animal Care and Use Committee (IACUC) from Stockholm (Sweden) or Lisbon (Portugal), and conducted in accordance with Portuguese and Swedish legislation on animal care and the European Community guidelines (Directive 2010/63/EU).

Most of the experiments were performed using male C57Bl6/J mice, aged between 8 and 18 weeks (most frequently 9–13 weeks) (Charles River Laboratories, Paris). In some cases, male and female mice were used to maximize the use of A1R knockout mice; because of this, control experiments using male and female mice have been performed. No appreciable differences between data obtained in males or females were detected (**Supplementary Figure S1**). The adenosine A_1 receptor knockout ($A_1R^{-/-}$) and wild-type ($A_1R^{+/+}$) mice were generated by inactivating the second protein coding exon of the mouse A_1R gene, from heterozygous breeding pairs with C57Bl6/J background strain (Johansson et al., 2001), obtained from a breeding colony derived from this original line that is housed at Karolinska Institutet, Sweden, and genotyped as described previously (Yang et al., 2015). All animals were social housed under standardized conditions of light (12-h light/12-h dark cycle), temperature (22–24°C), humidity (55–65%), and environmental enrichment (cardboard tubes plus nest material) and had free access to food and tap water.

Hippocampal Slices

Hippocampal slices were prepared as previously (e.g., Diógenes et al., 2004). The animals were sacrificed by decapitation under deep isoflurane anesthesia. The hippocampus was dissected free within ice-cold artificial cerebrospinal fluid (aCSF) solution composed of (millimeter): NaCl 124, KCl 3, $NaHCO_3$ 26, Na_2HPO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2; and glucose 10, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400-μm thick) were cut perpendicularly to the long axis of hippocampus with a McIlwain tissue chopper and allowed to recover functionally and energetically for 1 h in a resting chamber filled with the same solution, at room temperature and continuously gassed.

Extracellular Recordings

For electrophysiological recordings of field excitatory post-synaptic potentials (fEPSP), individual slices were transferred into a submerged recording chamber (dual submerged chamber) over the nylon mesh and continually superfused with gassed aCSF solution at a constant flow (3 ml/min) and temperature (32°C). This allows oxygenation in both slice surfaces while permitting a relatively fast flow rate to facilitate drug replacement. Stimulation (rectangular 0.1 ms pulses, once every 20 s) was delivered through a concentric electrode placed on Schaffer collateral-commissural fibers, in the stratum radiatum near the CA3–CA1 border. The intensity of the stimulus was set to the one eliciting near 50% of the maximal response, and was maintained throughout the experiment except in those experiments designed to perform input–output curves. In such experiments, after a

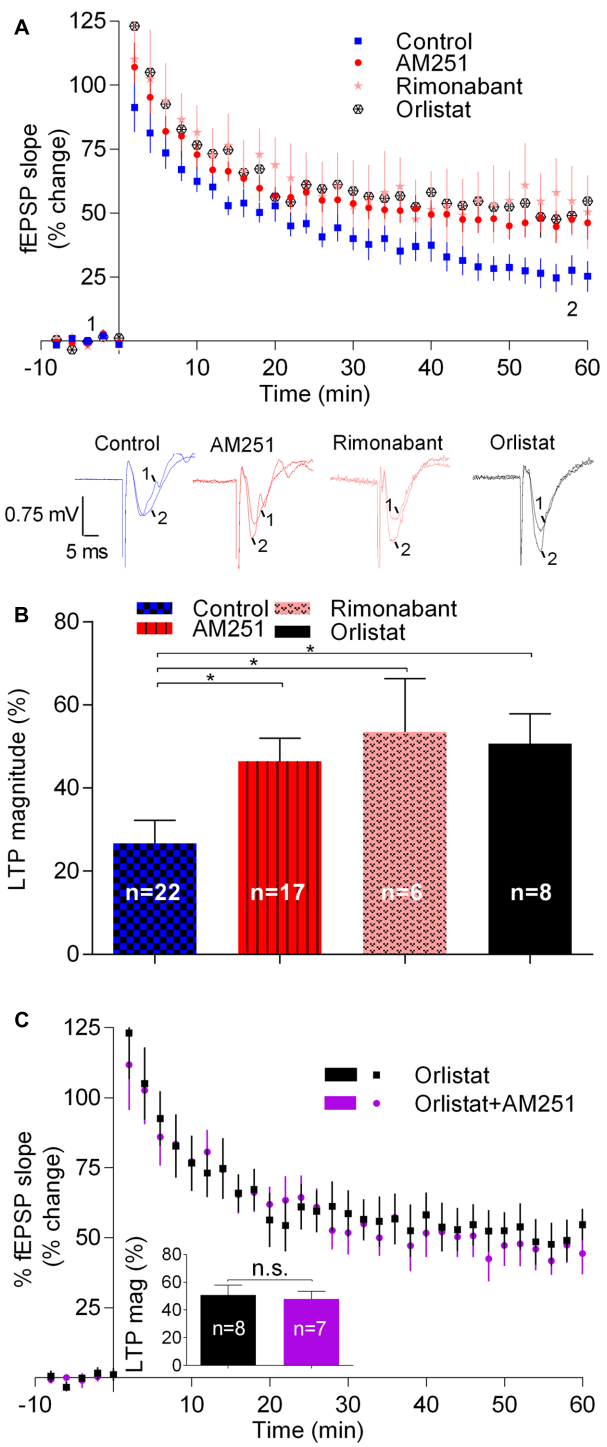

FIGURE 1 | Endocannabinoids inhibit LTP induced by weak-θ-burst stimulation (five trains of 100 Hz, 4 stimuli, separated by 200 ms). **(A)** Time course of the averaged fEPSP slopes in control conditions (no drugs) or in the presence of 1 μM AM251 (CB_1R inverse agonist), 1 μM Rimonabant (CB_1R antagonist), or 10 μM Orlistat (a fatty acid synthesis inhibitor). Data are represented as % of the averaged fEPSP slope recorded for 10 min before LTP induction, which were taken as zero%. Original traces taken from representative individual experiments and recorded during the baseline (1) and *(Continued)*

FIGURE 1 | Continued
50–60 min after weak-θ-burst induction (2) are shown below the time course panel. Each trace is composed by the stimulus artifact, followed by the presynaptic volley and the fEPSP. **(B)** Quantification of LTP magnitude under the indicated drug conditions. LTP magnitude was quantified as the % increase in fEPSP slope recorded at the 50–60 min after LTP induction, compared to the value recorded during the 10 min immediately before LTP induction; zero% represents no LTP and 100% would correspond to fEPSP slopes (at 50–60 min after LTP induction) twice the value recorded before LTP induction. $^*p < 0.05$ ($F_{(4,51)} = 2.986$, one-way ANOVA with Sidak's correction). **(C)** Non-additivity of the facilitatory action of AM251 and Orlistat, when added together. Data are represented as time course of fEPSP slopes and inset shows average LTP magnitude (LTP mag, defined as in **B**) in the two conditions, the color of the bars corresponding to the color of the symbols in the time course. Data for Orlistat in **(A)** and **(C)** (time course) and in **(B)** and **(C)** (LTP magnitude) are repeated to allow comparison between the action of Orlistat in the absence or presence of AM251. All values are mean ± standard error of mean (SEM) from *n* experiments; *n* values are indicated on the bars. $F_{(7,6)} = 1.8$, ns: $p > 0.05$ (Student's *t*-test).

stabilization period under the standard stimulation conditions, the stimulus intensity was increased by 20 μA every 6 min, within a range of 80–300 μA. fEPSP recording was through a microelectrode filled with NaCl 4 M (2–6 MΩ resistance), placed in CA1 stratum radiatum, coupled to an Axoclamp 2B Amplifier (Axon Instruments) and digitized BNC-2110 (National Instruments). Individual responses were monitored, and averages of six consecutive responses were continuously stored on a personal computer with the WinLTP Software (Anderson and Collingridge, 2001). fEPSPs were continuously recorded under basal stimulation frequencies and LTP was induced only after obtaining stable fEPSP slope values for at least a 15 min. Test drugs were added to the perfusing aCSF at least 30 min before LTP induction, or initiation of the input–output curves. Changes in stimulus frequency (LTP induction) or intensity (input–output curves) were only initiated after at least a 15 min stable baseline in the presence of the drugs.

Long-term potentiation was induced by θ-burst stimulation. Two different stimulation paradigms were used in different experiments, weak-θ-burst and strong-θ-burst protocols, which differed only in the number of trains delivered. The weak-θ-burst consisted of five trains whereas the strong-θ-burst was composed of 10 trains, in both cases the stimulation trains were separated by 200 ms. In both paradigms each train was composed of four stimuli delivered at 100 Hz. LTP magnitude was quantified as the % change in the average fEPSP slopes recorded from 50 to 60 min after LTP induction, taking as 0% the averaged fEPSP slope recorded for 10 min immediately before LTP induction. Throughout the text, while referring to weak LTP or to strong LTP we mean LTP induced by a weak-θ-burst or by a strong-θ-burst, respectively.

Drugs

The following drugs were used: WIN55,212-2 (WIN) mesylate, AM251, 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX), picrotoxin (PTX), JZL 184, and JZL 195 from Tocris. SR141716A (Rimonabant), tetrahydrolipstatin (Orlistat) from Biogen,

URB597 from Cayman Chemicals, barium salt of DL-fluorocitric acid from Sigma-Aldrich.

WIN55,212-2 was used as CB_1R agonist at a concentration (500 nM) 250 times higher than its K_i value for these receptors (Kuster et al., 1993). AM251 was used as a CB_1 receptor inverse agonist at a concentration (1 μM) 100 times higher than its K_i value for these receptors (Lan et al., 1999). Rimonabant was used as a CB_1 receptor antagonist at a concentration (1 μM) 500 times higher than its K_i for this receptor (Rinaldi-Carmona et al., 1994). DPCPX was used as an adenosine A_1 receptor antagonist at a concentration (50 nM) 100 times higher than its K_i value for this receptor (Bruns et al., 1987). PTX was used as a $GABA_A$ receptor antagonist at a concentration (50 μM) 100 times higher than its K_i value this receptor (Mehta and Ticku, 2013). JZL 184 was used as potent and selective monoacylglycerol lipase (MAGL) inhibitor at concentration (1 μM) 125 times higher than its IC_{50} for this enzyme (Long et al., 2009a). JZL 195 was used as potent inhibitor of both fatty acid amide hydrolase (FAAH) and of MAGL at a concentration (1 μM), respectively, 500 and 250 times higher than its IC_{50} for these enzymes (Long et al., 2009b). Orlistat was used as a diacylglycerol (DAG) lipase inhibitor at a concentration (10 μM) 100 times higher than the IC_{50} to inhibit DAG lipases α (Bisogno et al., 2006). URB597 was used as a selective FAAH inhibitor at a concentration (1 μM) 200 times the IC_{50} to inhibit this enzyme (Kathuria et al., 2003). Care was taken to use drug concentrations within selectivity ranges and according to previously published work using the same drugs for similar purposes. Solutions of all these drugs were prepared as stock solutions in 100% dimethylsulfoxide (DMSO). Aliquots of these stock solutions were diluted in aCSF in the day of the experiment. The concentration of the stock solution was chosen so that the final concentration of DMSO in the perfusion solutions was ≤0.1% (v/v).

Sodium fluorocitrate, an astrocyte metabolism inhibitor (Bonansco et al., 2011), was prepared as described by Paulsen et al. (1987): 8 mg of the barium salt of DL-fluorocitric acid was dissolved in 0.1 M HCl, precipitated by the addition of 0.1 M Na_2SO_4, buffered with 0.1 mM Na_2HPO_4 and centrifuged at 1000 × *g* for 5 min; the supernatant containing fluorocitrate was added to aCSF at a final concentration of 200 μM (pH 7.4).

Statistical Analysis

Data are expressed as the mean ± SEM; *n* corresponds to the number of experiments; in each experiment, only one slice was used per drug condition. At least one drug condition and the corresponding control was tested in each experimental day. Statistical significance was assessed by two-tailed Student's *t*-test when comparing two groups, or by one-way ANOVA with treatment as the between-subject factor, followed by Sidak's *post hoc* test when comparing multiple experimental groups. For the input–output curves, statistical significance was assessed by two-way ANOVA with treatment as the between-subject factor, followed by Sidak's *post hoc* test when comparing multiple experimental groups. A *p*-value of <0.05 was considered to account for significant difference. Analyses were performed with the GraphPad Prism 6 Software.

RESULTS

Physiologically Released Endocannabinoids Reduce LTP Induced by a Weak-θ-Burst

The first series of experiments was designed to evaluate the influence of eCBs upon weakly induced LTP. The influence of eCBs was assessed by testing the consequences of drugs that prevent CB_1R activation by eCBs or the synthesis of eCBs. We focused upon the synthesis of a predominant eCB at the hippocampus, 2-AG (Piyanova et al., 2015).

In control slices, fEPSP slopes recorded 50–60 min after inducing LTP with a weak-θ-burst, were 26.7 ± 5.5% higher than before LTP induction ($n = 18$; **Figure 1**). In slices where the CB_1R inverse agonist, AM251 (1 μM) was added to the perfusion at least 30 min before LTP induction, the magnitude of LTP was 46.5 ± 5.4% ($n = 17$, $t = 2.6$, $p < 0.05$ vs. control, **Figure 1**), which corresponds to near 80% increase in LTP magnitude. A similar result was obtained in the presence of another CB_1R blocker, the selective CB_1R antagonist, rimonabant (1 μM) (LTP magnitude: 53.5 ± 12.8%, $n = 6$, $t = 2.5$, $p < 0.05$ vs. control, **Figure 1**). In the presence of Orlistat (10 μM), an inhibitor of DAG lipase, the enzyme responsible for the conversion of DAG into 2-AG, the magnitude of LTP was also enhanced toward 50.7 ± 7.2% ($n = 8$, $t = 2.5$, $p < 0.05$; **Figure 1**). Importantly, when both CB_1R activation and 2-AG synthesis were prevented together, by the simultaneous presence of AM251 (1 μM) and Orlistat (10 μM) the magnitude of LTP was enhanced at the same degree as obtained with each of the drugs alone ($t = 0.3$, $p > 0.05$, **Figure 1C**). This lack of additivity indicates that both drugs facilitate LTP due to their common ability to prevent eCB signaling.

Summarizing, the above results show that drugs known to prevent the activation of CB_1R by eCBs or drugs known to inhibit the synthesis of 2-AG, the predominant eCB in the hippocampus (Piyanova et al., 2015), cause a marked facilitation of LTP induced by a weak-θ-burst, thus suggesting that eCBs inhibit such form of LTP.

Physiologically Released Endocannabinoids Enhance LTP with a Strong-θ-Burst

We then assessed the influence of eCBs on LTP induced by a strong-θ-burst protocol, all other experimental conditions being similar to those used before. Fifty–sixty minutes after the strong-θ-burst stimulation, LTP magnitude in control conditions was 68.1 ± 3.7% ($n = 22$) of pre-θ-burst stimulation. LTP dropped off by around 40% in the presence of AM251 (29.6 ± 6.8%, $n = 9$, $t = 5.1$, $p < 0.001$; **Figure 2**) or of rimonabant (28.5 ± 7.4%, $n = 5$, $t = 4.2$, $p < 0.01$; **Figure 2**). In the presence of Orlistat, the magnitude of LTP also decreased toward similar values (30.3 ± 8.4%, $n = 5$, $t = 4.0$, $p < 0.01$; **Figure 2**). It is worthwhile to note that in what concerns to the inhibition of LTP induced by a strong-θ-burst, the effect of AM251 was also not additive with that of Orlistat. Indeed, when both drugs were present, the LTP

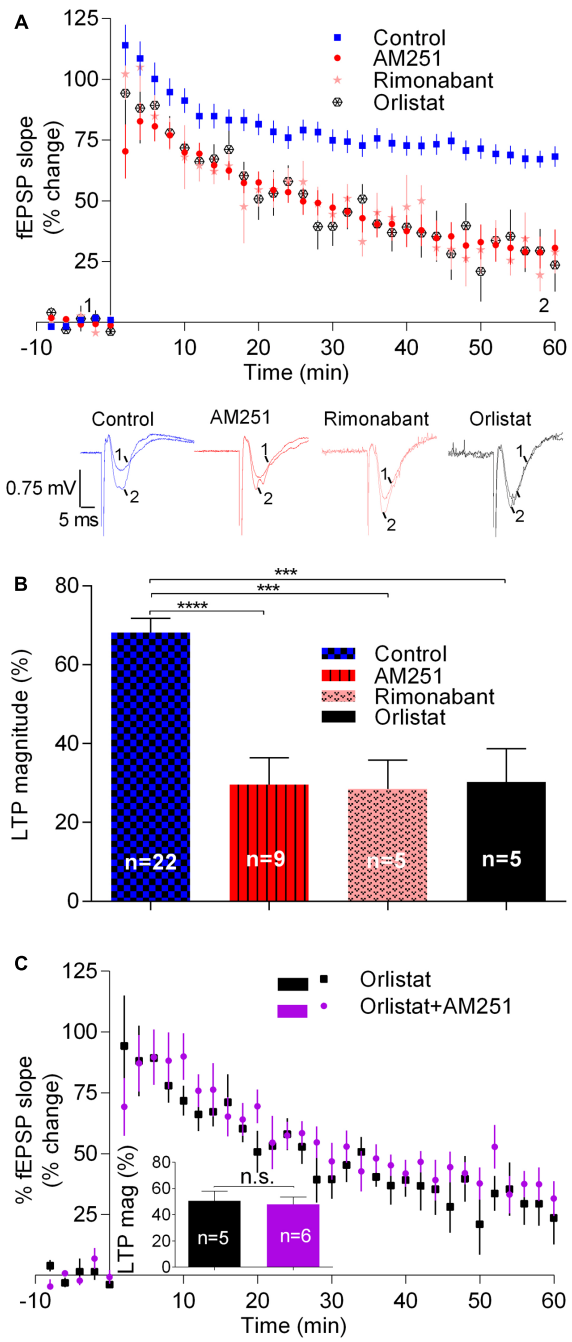

FIGURE 2 | Endocannabinoids enhance LTP induced by strong-θ-burst stimulation (10 trains of 100 Hz, 4 stimuli, separated by 200 ms). **(A)** Time course of the averaged fEPSP slopes, and original traces of fEPSP recordings, in control conditions (no drugs) or in the presence of 1 μM AM251 (CB_1R inverse agonist), 1 μM Rimonabant (CB_1R antagonist), or 10 μM Orlistat (a fatty acid synthesis inhibitor). **(B)** Quantification of LTP magnitude under the indicated drug conditions. $^{**}p < 0.01$; $^{***}p < 0.001$ ($F_{(7,91)} = 11.0$, one-way ANOVA with Sidak's correction). **(C)** Non-additivity of the inhibitory effect of AM251 and Orlistat, when added together. ns: $p > 0.05$ ($F_{(4,5)} = 1.4$, Student's *t*-test). Data for Orlistat in **(A)** and **(C)** (time course) and in **(B)** and **(C)** (LTP magnitude) are repeated to allow comparison between the action of Orlistat in the absence or presence of AM251. For further details see legend to **Figure 1**.

magnitude was 38.5 ± 6.4% ($n = 6$), a value significantly different ($t = 0.8$, $p < 0.05$) from that obtained in control conditions, but of similar magnitude as that obtained in the presence of each of the drugs separately (**Figure 2**). Again, this suggests that the ability of these drugs to inhibit strongly induced LTP results from their common ability to prevent eCB signaling.

Summarizing, the results reported in this section show that drugs known to prevent CB_1R activation by eCBs or to inhibit 2-AG synthesis lead to an inhibition of LTP induced by strong-θ-burst. These data are in clear contrast with what was observed when inducing LTP with a weak-θ-burst, and suggest that LTP induced by a strong-θ-burst is facilitated by eCBs.

Continuous Stimulation of CB_1R or the Non-prevalent eCB Leads to LTP Inhibition

The approach described in the previous sections was always directed toward the consequences of preventing CB_1R activation by eCBs. On the light of what is known about the inhibitory action of cannabinoids on neuronal activity, our finding that LTP induced by a strong-θ-burst is reduced by preventing CB_1R activation was unexpected. We thus decided to assess how this form of LTP is affected by continuous activation of CB_1Rs. To do so we used two approaches: (1) test the influence of inhibitors of eCB hydrolysis and in such way create conditions for sustained enhanced levels of eCBs, or to (2) use a CB_1 receptor agonist to exogenously activate CB_1Rs in a sustained way.

Data shown in **Figure 3** summarize the findings while using inhibitors of enzymes that prevent hydrolysis of eCBs. When using JZL 184 (1 μM), a selective inhibitor of MAGL, the enzyme that hydrolyses 2-AG, the magnitude of LTP was enhanced toward 92.3 ± 11.2% ($n = 9$, $t = 2.6$, $p < 0.05$ as compared with absence of drugs, **Figure 3**), corresponding to a value about 40% higher than that obtained in the absence of any drug. This finding suggests that enhancement of the levels of the predominant eCB in the hippocampus, 2-AG (Piyanova et al., 2015), facilitates strong LTP, thus in line with previous results showing that blockade of CB_1R or inhibition of synthesis of 2-AG inhibit strong LTP.

Remarkably, in the presence of URB 597, which at the concentration used (1 μM) inhibits FAAH, but not MAGL, the magnitude of LTP decreased toward 33.3 ± 8.6% ($n = 7$, $t = 3.4$, $p < 0.05$ as compared with absence of drugs, **Figure 3**), thus toward near half of the value obtained in control conditions. Since FAAH hydrolyses anandamide, this data suggest that accumulation of the non-predominant eCB in the hippocampus, anandamide (Piyanova et al., 2015), inhibits LTP in clear contrast with what occurs with the influence of the most abundant eCB in the hippocampus, 2-AG. This conclusion is further supported by the experiments where a non-selective inhibitor of both enzymes, FAAH and MAGL, was used. Thus, in the presence of JZL 195 (1 μM), the LTP magnitude was decreased toward a value (44.2 ± 8.8%, $n = 8$, **Figure 3**) between that obtained with URB 597 and that obtained in the absence of any drug, being not significant different ($t = 2.4$, $p > 0.05$) from any of these conditions. Altogether, the data with JZL 184, URB 597, and

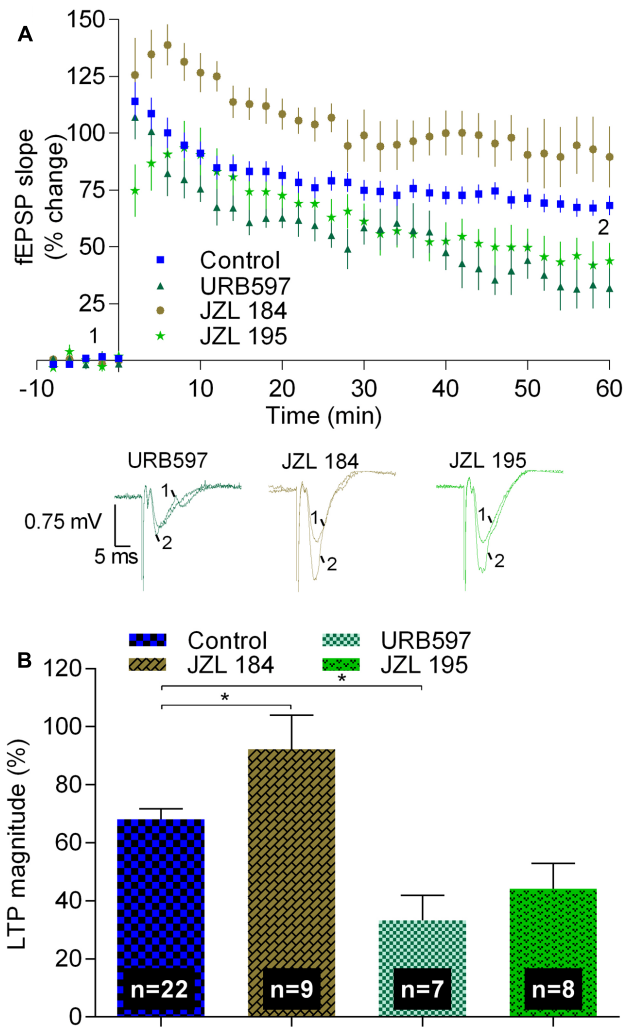

FIGURE 3 | Inhibitors of the hydrolysis of 2-AG or of anandamide differently affect strong-θ-burst induced LTP. **(A)** Time course of the averaged fEPSP slopes, and original traces of fEPSP recordings, in control conditions (no drugs) or in the presence of 1 μM JZL 184 (inhibitor of the enzyme that hydrolyses 2-AG, MAGL), 1 μM URB597 (inhibitor of the enzyme that hydrolyses anandamide, FAAH), or in the presence of 1 μM JZL 195 (inhibitor of FAAH and MAGL). **(B)** Quantification of LTP magnitude under the indicated drug conditions. Data in control conditions, in panels **(A)** and **(B)**, are the same as shown in **Figures 2A,B**, but are represented in this figure to allow comparison with the drug conditions. $^{*}p < 0.05$ ($F_{(3,42)} = 10.3$, one-way ANOVA with Sidak's correction). For further details see legend to **Figure 1**.

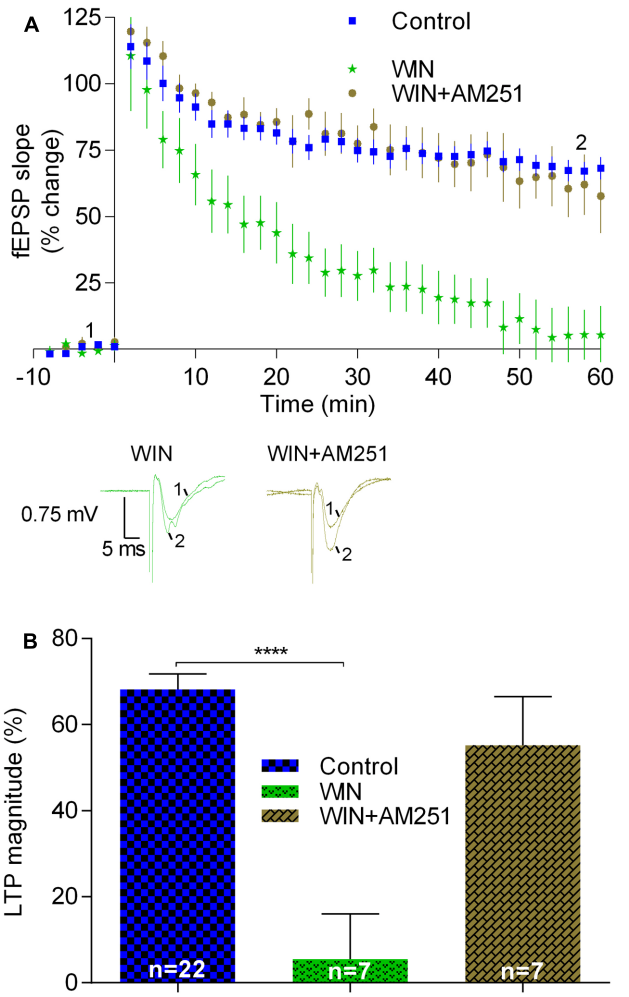

FIGURE 4 | LTP induced by strong-θ-burst is inhibited when CB_1Rs are continuously activated. **(A)** Time course of the averaged fEPSP slopes, and original traces of fEPSP recordings, in control conditions (no drugs) or in the presence of 500 nM WIN (CB_1R agonist), or 500 nM WIN together with 1 μM AM251. **(B)** Quantification of LTP magnitude under the indicated drug conditions. Data in control conditions in panels **(A)** and **(B)** are the same as shown in **Figures 2A,B**, but is represented in this figure to allow comparison with the drug conditions. $^{****}p < 0.0001$ ($F_{(2,33)} = 10.3$, one-way ANOVA with Sidak's correction). For further details see legend to **Figure 1**.

JZL 195 allow to suggest that enhanced production of 2-AG and enhanced production of anandamide affect strong LTP in an opposed way. However, the possibility that the inhibitory action of URB 597 results from non-CB1-related mechanisms (Kathuria et al., 2003; Ratano et al., 2017) cannot be fully excluded.

Secondly, we tested the effect of WIN (500 nM), a compound known to activate CB_1R. Since the effect of WIN upon synaptic transmission is known to be rather slow (Serpa et al., 2009), the slices were pre-incubated with WIN for at least 60 min before transfer to the acquisition chamber. Then, the slices were stabilized for at least 20 min, LTP being only induced when fEPSP slope values remained stable for at least 15 min. In such experiments LTP was virtually abolished (LTP magnitude: 5.5 ± 10.5%, $n = 7$, $t = 6.5$, $p < 0.05$ vs. pre-LTP induction; $p < 0.0001$ vs. LTP magnitude in control conditions; **Figure 4**). This inhibitory effect of WIN was prevented when the slices had been pre-incubated with the CB_1R inverse agonist, AM251, before addition of WIN. Indeed, under such conditions the inhibitory effects of both the agonist and the antagonists seem to be reciprocally canceled since LTP magnitude obtained in slices in the presence of AM251 and WIN (55.2 ± 11.2%, $n = 7$, **Figure 4**) was similar ($t = 1.3$, $p > 0.05$) to that obtained in the absence of any drug.

Summarizing, the data reported in this section suggest that sustained activation of CB_1Rs induced by adding an exogenous agonist as well as prevention of degradation of the non-prevalent eCB in the hippocampus leads to inhibition of LTP induced by the strong-θ-burst. This is in clear contrast with the conclusions that could be drawn while assessing the action of a drug known to prevent the hydrolysis or prevent the formation of the predominant eCB in the hippocampus as well as when accessing the action of transiently released eCBs by using CB_1R blockers. Altogether, the data indicate that while physiologically released eCBs are required to facilitate LTP induced by a strong θ-burst, non-physiological activation of CB_1R leads to inhibition of this form of LTP.

Inhibition of LTP during CB_1R Blockade Is Not a Result of Enhanced A_1R Activation

The above results indicating that physiologically released eCBs can facilitate LTP lead us to hypothesize that the strong-θ-burst could lead to the recruitment of other neuromodulators that would affect the neuromodulatory influence of eCBs. Purines are released during high-frequency neuronal firing (Cunha et al., 1996) and adenosine is known to inhibit LTP through activation of A_1R (De Mendonça and Ribeiro, 2000; Wang et al., 2016), which are abundantly expressed in the hippocampus. Furthermore, A_1R can affect CB_1R signaling (Hoffman et al., 2010; Sousa et al., 2011). To test if the apparent facilitatory action of eCBs upon strong-θ-burst-induced LTP could be due to any interference with endogenous adenosine acting on A_1R, we used two different approaches: genetic ($A_1R^{-/-}$ mice) or pharmacological (selective A_1R antagonist, DPCPX) prevention of A_1R activity.

The magnitude of LTP induced by the strong-θ-burst in slices from $A_1R^{-/-}$ mice (65.5 ± 6.7%, n = 13) was not significantly different (t = 0.35, p > 0.05) from that obtained in $A_1R^{+/+}$ (68.1 ± 3.7%, n = 22, **Figure 5**). Remarkably, the CB_1R inverse agonist AM251 inhibited LTP toward a similar value in both genotypes [$A_1R^{+/+}$: 29.6 ± 6.8%, n = 9; $A_1R^{-/-}$: 25.1 ± 9.3%, n = 8, t = 0.4, p > 0.05 when comparing genotypes; t = 4.1, p < 0.05 when assessing the effect of AM251 in $A_1R^{-/-}$ (control $A_1R^{-/-}$ vs. $A_1R^{-/-}$: AM251), **Figure 5**]. These data suggest that the inhibition of strong LTP caused by the inverse agonist of CB_1R does not result from an enhanced A_1R activation by released adenosine. To further confirm this, and to preclude any adaptation-like process due to genetic removal of A_1R, we tested action of AM251 in $A_1R^{+/+}$ mice following inhibition of the A_1R with DPCPX. Again, and in spite the presence of DPCPX at a concentration (50 nM) near 100 times its K_i for A_1R (Bruns et al., 1987), thus expected to fully block A_1R signaling, AM251 caused a marked inhibition of LTP induced by the strong-θ-burst (DPCPX: 68.0 ± 9.3%, n = 9; DPCPX+AM251: 32.5 ± 5.2%, n = 7, t = 3.3, p < 0.05, **Figure 5**).

Altogether the above data seem to indicate that the inhibition of LTP caused by preventing CB_1R activation by eCBs is not due to enhanced activation of A_1R by endogenous adenosine.

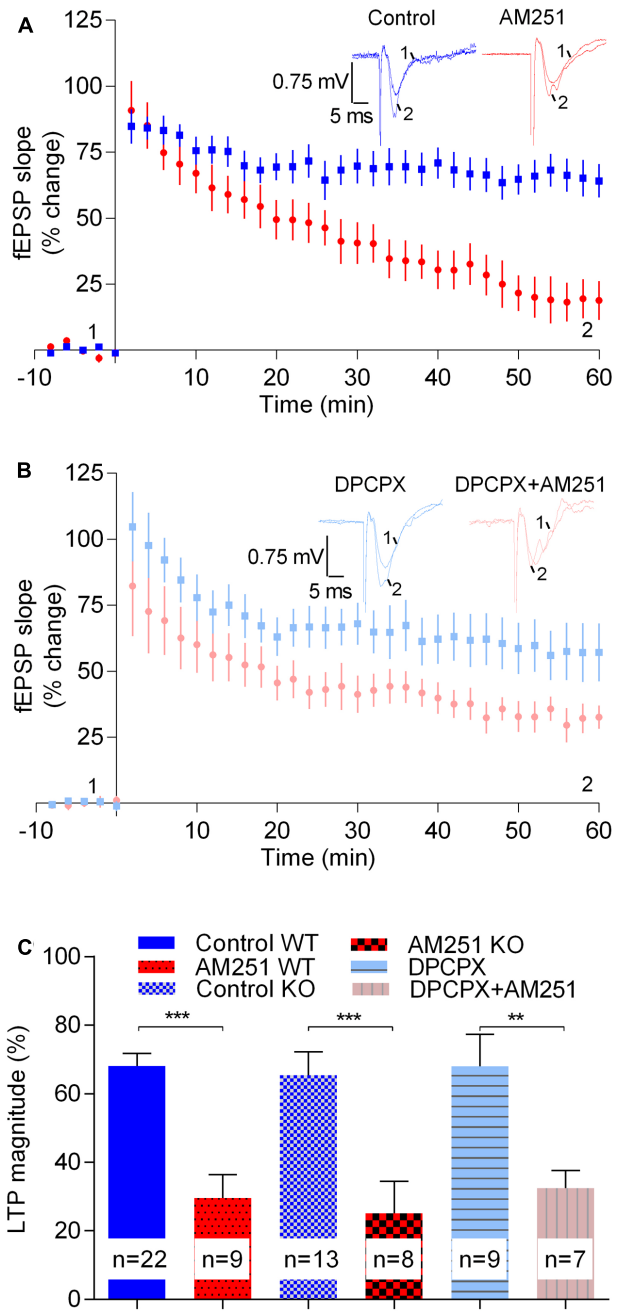

FIGURE 5 | The enhancement LTP caused by physiologically released eCBs does not result from enhanced adenosinergic tonus on A_1R. **(A)** Data obtained in slices taken from A_1R KO mice, LTP being induced by a strong-θ-burst in control conditions (no drugs, blue symbols and traces) or in the presence of 1 μM of AM251 (red symbols and traces). **(B)** Data obtained in slices from wild-type mice in the presence of the A1R antagonist, DPCPX (50 nM) either in the absence (blue symbols and traces) or presence (pink symbols and traces) of AM251 (1 μM). **(C)** Quantification of LTP magnitude under the indicated conditions. In all cases LTP was induced by a strong-θ-burst. Data from WT mice in the absence of DPCPX (control WT, AM251 WT, panel **C**) are the same as that shown in **Figure 2B**, but is represented in this figure to allow comparisons with data from A_1R KO mice and with data from WT slices in the presence of DPCPX. $^{**}p$ < 0.01; $^{***}p$ < 0.001 ($F_{(5,62)}$ = 10.0, one-way ANOVA with Sidak's correction). For further details see legend to **Figure 1**.

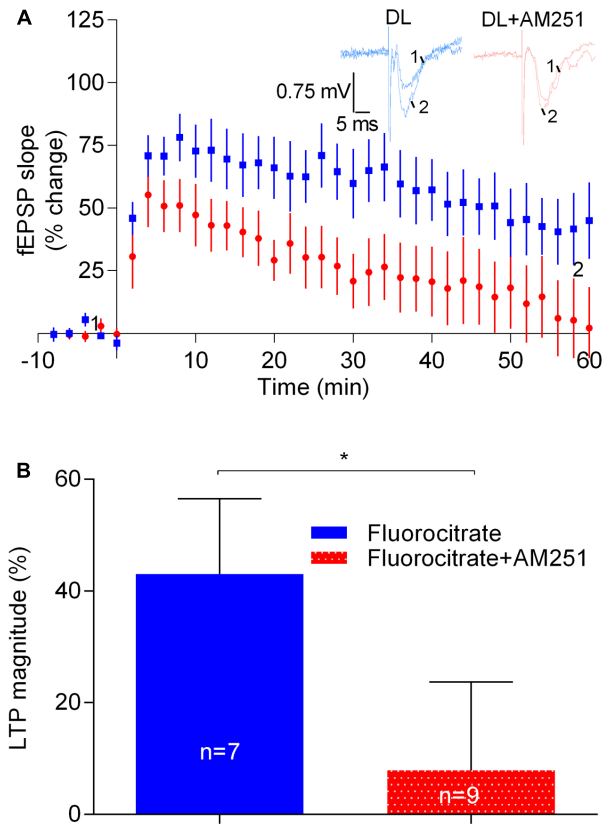

FIGURE 6 | Astrocytes do not contribute to the enhancement of LTP caused by physiologically released eCBs. **(A)** Data obtained in slices treated with 200 μM Fluorocitrate to inhibit astrocyte metabolism, either in the absence (control) or in the presence of 1 μM of AM251. **(B)** Quantification of LTP magnitude under the indicated conditions. In all cases, LTP was induced by a strong-θ-burst. $*p < 0.05$ ($F_{(8,6)} = 1.9$, Student's *t*-test). For further details see legend to **Figure 1**.

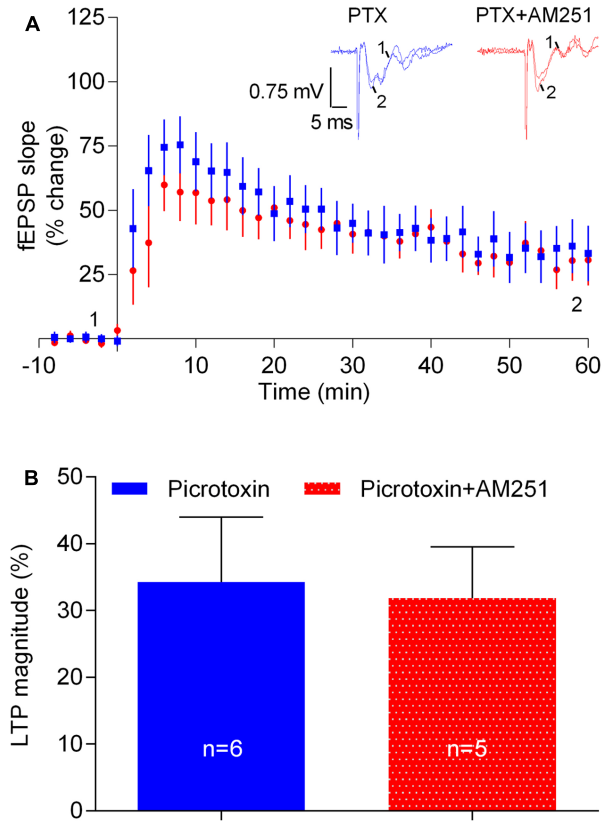

FIGURE 7 | The influence of physiologically released eCB upon strong-θ-burst-induced LTP is lost upon blockade of GABAergic transmission. **(A)** Data obtained in slices treated with the $GABA_A$ receptor antagonist, PTX (50 μM) in the absence (control) or in the presence of 1 μM of AM251. **(B)** Quantification of LTP magnitude under the indicated conditions. No significant differences between both conditions were found ($F_{(5,4)} = 1.9$, $p > 0.05$, Student's *t*-test). For further details see legend to **Figure 1**.

The absence of A_1Rs also did not affect the inhibitory action of the CB_1R agonist (500 nM WIN) upon LTP magnitude. Thus, in $A_1R^{(-/-)}$ mice the magnitude of LTP in the presence of 500 nM WIN (-0.57 ± 9.4, $n = 5$) was significantly lower ($t = 4.1$, $p < 0.0001$) than in the absence of WIN and not different ($t = 0.5$, $p > 0.05$) from LTP magnitude in slices from WT mice in the presence of 500 nM WIN.

Astrocytes Do Not Contribute to the Enhancement of LTP Caused by eCBs

Astrocytes are known to contribute to the facilitatory action of eCBs upon glutamatergic transmission (Navarrete and Araque, 2010). In addition, it is known that astrocytes, by releasing gliotransmitters, which then act in pre- and post-synaptic receptors, affect neuronal signaling and plasticity (Pascual et al., 2005; Henneberger et al., 2010). We thus hypothesized that the apparent facilitatory action of eCBs upon LTP induced by the strong-θ-burst would involve the astrocytes. To address that possibility we incubated the slices with the metabolic gliotoxin fluorocitrate (200 μM) for at least 20 min and allowed the fEPSP slopes to stabilize for at least 15 min before inducing LTP either in the presence or absence of AM251. As expected from previous reports (Bonansco et al., 2011) LTP magnitude was reduced in slices incubated with fluorocitrate (cf. data in **Figure 6** with **Figure 2**). Remarkably, however, under such conditions the CB_1R inverse agonist, AM251, was still able to markedly inhibit LTP (fluorocitrate: $43.0 \pm 13.5\%$, $n = 7$; fluorocitrate +AM251: $7.9 \pm 15.8\%$, $n = 9$, $t = 2.3$, $p < 0.05$ vs. fluorocitrate alone; **Figure 7**).

These data suggest that the apparent facilitatory action of eCBs upon strong-θ-burst-induced LTP does not involve the astrocytes. This data also show that, at least under some experimental conditions, the strength of LTP induction may be even more determinant of the direction of the influence of eCBs upon LTP than the magnitude of LTP itself.

The eCB-Mediated Enhancement of LTP Is GABAergic Transmission Dependent

Next we hypothesized that the apparent facilitatory action of eCBs upon LTP caused by the strong-θ-burst stimulation could

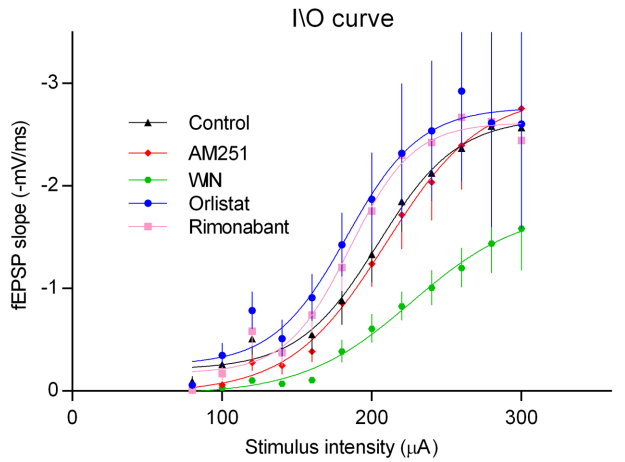

FIGURE 8 | Input/output curves where fEPSP slopes values are plotted against the stimulation intensities (80 and 300 μA) in hippocampal slices under the drug conditions indicated. All values in the figure are represented as the mean ± SEM, n = (5–8). Statistical significance was assessed by two-way ANOVA followed by Sidak's *post hoc* test, comparing multiple experimental groups; no significant differences among drug treatments were detected except for the WIN; $^*p < 0.05$ as compared with the other drugs) ($F_{interaction(44,360)} = 0.5$, $F_{row\ factor(11,360)} = 38.1$, $F_{column\ factor(4,360)} = 14.4$, t-values: control/AM251 – 0.6, control/WIN – 5.2, control/Rimonabant – 1.1, control/Orlistat – 2.1).

be due to a preponderant inhibition of GABA release over glutamate release. To test this possibility, experiments were performed in the presence of the $GABA_AR$ antagonist, PTX (50 μM). LTP magnitude was smaller in the presence of PTX, as compared with its absence (cf. **Figures 2**, **7**), which may result from overactivity of glutamatergic transmission even before LTP induction. Remarkably, in slices in the presence of PTX, the inhibitory action of AM251 upon LTP was lost (PTX: 34.3 ± 9.7%, n = 6; PTX+AM251: 31.8 ± 7.7%, n = 5; 0.1, $p > 0.05$, **Figure 7**). The ability of PTX to prevent the inhibitory action of AM251 upon LTP should not be attributed to its ability to diminish LTP, since fluorocitrate also inhibited LTP and did not prevent the action of AM251 (cf. **Figures 6**, **7**).

The above data suggest that the apparent facilitatory action of eCBs upon LTP induced by the strong-θ-burst involves $GABA_AR$-mediated GABAergic transmission, most probably resulting from eCB-induced inhibition of GABA release with consequent disinhibition of glutamatergic neurons.

Prevention of CB_1R Activation by eCBs Does Not Affect Basal Excitability

Long-term potentiation can be influenced by changes in basal synaptic transmission. To evaluate if manipulation of 2-AG signaling could have a global influence upon excitability, input/output (I/O) curves were compared in the absence and presence of AM251, Rimonabant, or Orlistat. As illustrated in **Figure 8**, none of these drugs appreciably modified I/O curves compared with the control. Exogenous activation of CB_1R using the CB_1R agonist WIN, did however clearly altered the I/O curve, an action that can be attributed to its well-known ability to inhibit synaptic transmission at the CA1 area of the hippocampus (Serpa et al., 2009). The absence of influence of AM251, Rimonabant, or Orlistat in I/O curves but their influence upon LTP, indicates that transiently released 2-AG have a predominant influence over LTP rather than over basal synaptic transmission.

DISCUSSION

A main finding in the present work is that prevention of CB_1R activation may affect CA1 LTP in an opposing way, depending on the strength of LTP induction and the magnitude of LTP itself. Thus, we show that a CB_1R inverse agonist, a CB_1R antagonist as well as an inhibitor of the formation of 2-AG, the main eCB in the hippocampus (Piyanova et al., 2015), leads to a facilitation of weakly induced LTP but to an inhibition of strongly induced LTP. This suggests that eCBs inhibit weak LTP while facilitating a more robust LTP. In accordance to the idea that physiologically released eCBs favor robust LTP is also the finding that an inhibitor of the degradation of 2-AG facilitates LTP induced by the strong-θ-burst. However, continuous activation of CB_1R with an exogenous agonist or overproduction of a non-prevalent eCB, anandamide, leads to inhibition of strongly induced LTP. Overall, these findings are suggestive of dual actions of eCBs upon CA1 LTP depending both on the strength of LTP induction as well as on the nature of CB_1R activators.

To our knowledge, this is the first time that dual action of cannabinoids upon hippocampal LTP is clearly shown. It is known for a long time that mice lacking CB_1Rs have enhanced hippocampal LTP (Bohme et al., 1999; Jacob et al., 2012), compatible with the general idea of inhibitory actions of cannabinoids in the brain. Similarly, the work by Slanina et al. (2005) clearly showed that weak LTP, induced by a small number of pulses delivered at the CA1 area of hippocampal slices, is facilitated by CB_1R blockade, also allowing the suggestion that eCBs inhibit weakly induced LTP. However, LTP induced by strong non-θ-burst high frequency stimulation (100 pulses for 1 s, or twice this paradigm separated by 20 s) was unaffected by CB_1R blockade (Slanina et al., 2005), in clear contrast with the results herein reported for robust θ-burst-induced LTP. Species differences (mice in our study vs. rats in the study by Slanina et al., 2005) or age of the animals (adults in our study vs. adolescents in the study by Slanina et al., 2005) may account for these differences.

Facilitation of LTP by eCBs has been also reported, but again, those studies do not highlight a dual action of eCBs as a function of LTP magnitude. First evidence that eCBs facilitate CA1 hippocampal LTP was provided by the report that eCBs enable LTP induction by trains of EPSPs that are ineffective if eCBs are not allowed to act (Carlson et al., 2002). This action could be attributed to a eCB-mediated inhibition of GABAergic synapses (Carlson et al., 2002), and indeed it was later reported that upon removal of synaptic inhibition in a restricted area of the dendritic tree, there is a selective priming of nearby excitatory synapses by eCBs, which facilitate induction of CA1 hippocampal LTP (Chevaleyre and Castillo,

2004). Those studies allowed to understand the action of eCBs at the local circuitry and at the single neuron level, but do not inform on the global impact of eCBs upon LTP of pyramidal neurons. Using adult rats, De Oliveira Alvares et al. (2006) reported a marked inhibition of non-θ-burst high-frequency-induced CA1 LTP of fEPSPs by AM251, thus pointing that eCBs are required for robust LTP phenomena. More recently, Wang et al. (2016) reported that 2-AG and CB_1R signaling is required for LTP of the lateral perforant path input to dentate gyrus neurons. In the study by Wang et al. (2016), however, robustly induced CA1 LTP was unaffected by preventing CB_1R activation. In contrast, our data clearly point toward a facilitatory action of 2-AG and CB_1R signaling on CA1 LTP induced by robust stimulation. Pattern of stimulation (θ-burst in both cases), or age (adult animals in both cases), cannot account for the differences. The difference may reside in the characteristics of the perfusion chamber, which may impact upon the accumulation of endogenous substances around the synapses. We used a slice submersion chamber, while Wang et al. (2016) used an interface chamber; submerging chambers likely favor the accumulation of endogenous substances. Lower level of oxygenation in submerged chambers may, in some studies, account for differences between data obtained in submerged or interface chambers (Hájos and Mody, 2009). However, this might not be the case since our chambers are provided with nylon mesh thus allowing oxygenation in both surfaces of the slice. Under our experimental conditions the oxygen pressure in the perfusion solution inside the chamber is 500–600 mmHg (Sebastiao et al., 2001). We used mice while Wang et al. (2016) used rats when testing the influence upon CA1 LTP, but species differences are unlikely to account for the dissimilarities since no marked differences were detected by Wang et al. (2016) while comparing LPP–LTP data in mice and rat hippocampal slices.

Pyramidal hippocampal neurons are under inhibitory control of GABAergic synapses, but also under control of several modulatory substances. Adenosine, an ubiquitous molecule released by neurons and glia, is able to modulate synaptic transmission and plasticity by operating high affinity G-protein-coupled receptors (Sebastião and Ribeiro, 2015). The adenosine A_1R is highly expressed in the hippocampus and with a clear inhibitory action upon synaptic transmission and LTP (Dunwiddie and Masino, 2001; Boison, 2005; Sebastião and Ribeiro, 2015). The inhibitory action of CB_1R upon GABA and glutamate release, as well as on synaptic transmission in the hippocampus are partially reduced by co-activation of A_1R (Hoffman et al., 2010; Sousa et al., 2011), suggesting an interaction between these two modulatory pathways at the hippocampus (but see Serpa et al., 2009, 2015). The possibility that A_1R-mediated attenuation of an inhibitory effect of eCBs could justify the apparent excitatory action of eCBs upon strongly induced LTP led us to test if the action of a CB_1R blocker was affected by A_1R deletion or A_1R blockade. However, none of these significantly influenced the inhibitory action of AM251 upon strong LTP.

Astrocytes release several neuromodulatory substances, including purines (Henneberger et al., 2010; Lalo et al., 2014), and have been shown to contribute to the facilitatory action of eCBs upon hippocampal glutamatergic transmission (Navarrete and Araque, 2010). Metabolic inhibition of the astrocytes, a condition known to affect astrocytic signaling and release of gliotransmitters (Paulsen et al., 1987; Swanson and Graham, 1994; Bonansco et al., 2011) did, however, not affect the influence of AM251 upon LTP. This suggests that astrocytes do not play a major role in the facilitatory action of eCBs upon LTP.

A common conclusion in all studies reporting facilitation of LTP by eCBs is that it can be accounted by an influence upon GABAergic neurons (Carlson et al., 2002; Chevaleyre and Castillo, 2004; Wang et al., 2016). Accordingly, we also observed that the ability of the CB_1R blocker, AM251, to inhibit LTP was lost in the presence of the $GABA_AR$ antagonist, PTX, thus reinforcing the conclusion that physiologically released eCBs facilitate LTP by restraining the inhibition of LTP imposed by GABAergic inputs. It has previously been shown that deletion of CB_1R in GABAergic neurons leads to a decreased hippocampal CA1 LTP, whereas deletion of CB_1R in glutamatergic neurons leads to enhanced LTP (Monory et al., 2015). It is therefore likely that the two stimulation conditions used in the present work lead to a differential influence of eCBs in GABAergic interneurons and glutamatergic neurons, so that under strong LTP induction conditions the influence of eCBs upon GABAergic neurons predominates.

CB_1R are widely distributed in the central nervous system, mainly in the hippocampus, cortex, basal ganglia, and cerebellum (Marsicano and Lutz, 1999; Wilson and Nicoll, 2002). This receptor is localized in excitatory and inhibitory neurons (Katona et al., 2001; Wilson et al., 2001; Kawamura, 2006) and also in astrocytes (Hoffman et al., 2010; Han et al., 2012). Considering the neuronal compartment only, it has been estimated that about three quarters of all CB_1R present in hippocampi are on GABAergic neurons while glutamatergic neurons contain about one quarter of all hippocampal CB_1R (Steindel et al., 2013). Not all GABAergic hippocampal neurons express CB_1R, these receptors being localized in cholecystokinin (CCK) positive neurons. CCK-positive neurons express higher levels of CB_1R than the pyramidal cells (Marsicano and Lutz, 1999; Marsicano et al., 2003; Monory et al., 2006). It is thus not surprising that the apparent facilitatory action of eCBs upon strongly induced LTP results from an action upon GABAergic neurons, most probably by suppressing the inhibitory control exerted by CCK-positive basket cells over the pyramidal neurons.

Another relevant finding in the present work is the similarity between the effect of drugs that block CB_1Rs or inhibit formation of 2-AG, the predominant eCB in the hippocampus (Piyanova et al., 2015), and the effect of sustained activation of CB_1R by an exogenous agonist. It is worthwhile to note that the inhibitory action of Orlistat (2-AG synthesis inhibitor) and the inhibitory action of WIN (CB_1R agonist) were both counteracted by the CB_1R receptor blocker, AM251, clearly indicating that both are due to alterations in the level of CB_1R activation. It is known for a long time that the CB_1R activation inhibits LTP

(Nowicky et al., 1987; Collins et al., 1995; Terranova et al., 1995; Stella et al., 1997; Paton et al., 1998; Basavarajappa et al., 2014), these inhibitory actions being usually interpreted on the light of the knowledge that exocannabinoids inhibit excitatory synaptic transmission. However, the novelty of the present work is the possibility to contrast, under the same experimental conditions, the action of drugs that continuously activate CB_1R with those that reduce CB_1R activation, allowing to suggest that CB_1R can either facilitate or inhibit LTP as a function of several conditions, including the characteristics of CB_1R activation, the strength of LTP induction, as well as the magnitude of LTP itself. Also worthwhile to note is the contrast, under the same experimental conditions, between the influence of a drug known to inhibit hydrolysis the predominant eCB at the hippocampus, 2-AG, which facilitates strong LTP in line with the idea of a facilitatory action of endogenous activation of eCBs, with that of drugs that unselectively prevent eCB metabolism or that prevent metabolism of anandamide only, both of which inhibit strong LTP. These findings highlight differences in the modulatory actions of the eCBs, which may be relevant to interpret some age-dependent differences in the neuromodulatory actions of cannabinoids. Indeed, the relative abundance of anandamide over 2-AG increases throughout age (Piyanova et al., 2015). Our data thus contribute to interpret apparently discrepant data, and strongly support the idea of a dual action of eCB signaling to sustain LTP.

CONCLUSION

The data herein reported clearly show that manipulating eCB signaling may have opposing effects upon LTP, depending on the strength of LTP induction, inhibiting weak LTP and facilitating stronger LTP. This suggests that eCBs act as a high-pass filter, therefore likely reducing the signal-to-noise ratio of synaptic strengthening. Importantly, we also show that under the same LTP inducing conditions, prolonged activation of CB_1R with exocannabinoids or blockade of CB_1R may both impair LTP. Our data with drugs known to increase the accumulation of anandamide or 2-AG suggest that these two eCBs may differently affect LTP. Altogether, the data herein reported highlight a clear homeostatic control of eCBs and CB_1Rs upon LTP. Disruption of this finely tuned homeostatic role of eCBs upon synaptic plasticity phenomena likely underlies the known deleterious influence of cannabinoid-based drugs upon memory.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Directive 2010/63/EU." The protocol was approved by the "iMM's Institutional Animal Welfare Body – ORBEA-iMM and the National competent authority – DGAV (Direcção Geral de Alimentação e Veterinária)."

AUTHOR CONTRIBUTIONS

AS-C performed the experiments and quantified the data. AS and AS-C designed the experiments, analyzed, and discussed the data. MC provided breeding pairs for A1R knockout and WT mice. AS-C, MC, JR, and AS contributed to manuscript writing.

FUNDING

This work was supported by LISBOA-01-0145-FEDER-007391, project co-funded by FEDER through POR Lisboa 2020 (Programa Operacional Regional de Lisboa) from PORTUGAL 2020 and Fundação para a Ciência e Tecnologia (FCT), by an FCT project (PTDC/DTP-FTO/3346/2014), by Cardlane Ltd., by Twinning action (SynaNet) from the EU H2020 program (project number: 692340), by the Swedish Research Council (2016-01381; MC), the Swedish Heart and Lung Foundation (20140448; MC), and by Research Funds from the Karolinska Institutet. AS-C was in receipt of a Cardlane Ltd. fellowship.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2017.00921/full#supplementary-material

FIGURE S1 | No appreciable differences between data obtained in males or females were detected. Values are represented as the mean ± SEM. The number of experiments in each condition is indicated in the bars. No significant differences between males and females were found, $F_{(11,102)} = 7.2$, *t*-values – Control WT:Control WT male = 0.9, Control WT:Control WT female = 0.5, AM251 WT:AM251 WT male = 0.9, AM251 WT:AM251 WT male = 0.6, Control KO:Control KO male = 0.06, Control KO:Control KO female = 0.05, AM251 KO:AM251 KO male = 0.1, AM251 KO:AM251 KO female = 0.07. For further details on the way to calculate LTP magnitude see the section "Materials and Methods" and legend to **Figure 1**.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 02 February 2018
doi: 10.3389/fphar.2018.00047

Contributions of the Nucleus Accumbens Shell in Mediating the Enhancement in Memory Following Noradrenergic Activation of Either the Amygdala or Hippocampus

*Erin C. Kerfoot and Cedric L. Williams**

Division of Neuroscience and Behavior, Department of Psychology, University of Virginia, Charlottesville, VA, United States

The nucleus accumbens shell is a site of converging inputs during memory processing for emotional events. The accumbens receives input from the nucleus of the solitary tract (NTS) regarding changes in peripheral autonomic functioning following emotional arousal. The shell also receives input from the amygdala and hippocampus regarding affective and contextual attributes of new learning experiences. The successful encoding of affect or context is facilitated by activating noradrenergic systems in either the amygdala or hippocampus. Recent findings indicate that memory enhancement produced by activating NTS neurons, is attenuated by suppressing accumbens functioning after learning. This finding illustrates the significance of the shell in integrating information from the periphery to modulate memory for arousing events. However, it is not known if the accumbens shell plays an equally important role in consolidating information that is initially processed in the amygdala and hippocampus. The present study determined if the convergence of inputs from these limbic regions within the nucleus accumbens contributes to successful encoding of emotional events into memory. Male Sprague-Dawley rats received bilateral cannula implants 2 mm above the accumbens shell and a second bilateral implant 2 mm above either the amygdala or hippocampus. The subjects were trained for 6 days to drink from a water spout. On day 7, a 0.35 mA footshock was initiated as the rat approached the spout and was terminated once the rat escaped into a white compartment. Subjects were then given intra-amygdala or hippocampal infusions of PBS or a dose of norepinephrine (0.2 μg) previously shown to enhance memory. Later, all subjects were given intra-accumbens infusion of muscimol to functionally inactivate the shell. Muscimol inactivation of the accumbens shell was delayed to allow sufficient time for norepinephrine to activate intracellular cascades that lead to long-term synaptic modifications involved in forming new memories. Results show that memory improvement produced by infusing norepinephrine in either the amygdala or hippocampus is attenuated by interrupting neuronal activity in the shell 1 or 7 7 h following amygdala or hippocampus activation.

OPEN ACCESS

Edited by:
Antonella Gasbarri,
University of L'Aquila, Italy

Reviewed by:
Christa McIntyre,
The University of Texas at Dallas, United States
Almira Vazdarjanova,
Augusta University, United States

***Correspondence:**
Cedric L. Williams
clw3b@virginia.edu

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *25 September 2017*
Accepted: *15 January 2018*
Published: *02 February 2018*

Citation:
Kerfoot EC and Williams CL (2018) Contributions of the Nucleus Accumbens Shell in Mediating the Enhancement in Memory Following Noradrenergic Activation of Either the Amygdala or Hippocampus. Front. Pharmacol. 9:47. doi: 10.3389/fphar.2018.00047

These findings suggest that the accumbens shell plays an integral role modulating information initially processed by the amygdala and hippocampus following exposure to emotionally arousing events. Additionally, results demonstrate that the accumbens is involved in the long-term consolidation processes lasting over 7 h.

Keywords: emotions, memory, nucleus accumbens, norepinephrine, amygdala and hippocampus, inhibitory avoidance, memory modulation

INTRODUCTION

In response to new learning episodes, the shell division of the accumbens receives a constellation of neural input from brain regions that encode separate attributes of novel experiences. These inputs include signals from brainstem nuclei representing physiological changes induced by the event, information from the amygdala regarding the affective appraisal of stimuli and representations of contextual and temporal relationships of the episode from the ventral hippocampus (Mogenson et al., 1980; Groenewegen et al., 1987; Meredith et al., 1990; Wang et al., 1992; Brog et al., 1993; Petrovich et al., 1996; Delfs et al., 1998; Al'bertin, 2003; French and Totterdell, 2003; Jongen-Relo et al., 2003; McGinty and Grace, 2009). Interestingly, projections from the basolateral amygdala (BLA) and ventral hippocampus (HIPP) converge monosynaptically on projection neurons within the accumbens shell (French and Totterdell, 2003; Stuber et al., 2011; Britt et al., 2012). Neurons in the shell are depolarized following either high frequency or optogenetic stimulation of the BLA and these effects are suppressed by inactivating the HIPP (Gill and Grace, 2011; Correia et al., 2016). Based on this anatomical arrangement, it is suggested that information transmitted from either the amygdala or hippocampus in response to new learning may require extensive processing within the nucleus accumbens (Roozendaal et al., 2001; Correia et al., 2016). The accumbens shell therefore, may be in a critical position to modulate information emanating from limbic structures that encode separate features of newly experienced events.

Interactions between the basolateral amygdala, hippocampus and accumbens shell may involve unique roles in encoding the motivational and affective value of stimuli as well as contextual representations during new learning. Disrupting connections between the BLA and accumbens impairs the capacity of laboratory animals to acquire second-order conditioned responses (Setlow et al., 2002). Although rats with unilateral basolateral and contralateral accumbens lesions show no deficits in learning that a light stimulus signals food availability, the lesioned animals fail to acquire more complex associations such as learning that a tone presented before the light also signals food availability. Additionally, previous studies revealed that ventral hippocampus lesions impair memory for contextual fear conditioning, whereas dorsal lesions disrupt spatial learning in tasks such as the Morris water maze task (Richmond et al., 1999; Burhans and Gabriel, 2007; Ji and Maren, 2008; Czerniawski et al., 2009). Given that the ventral hippocampus projects to the accumbens shell (Groenewegen et al., 1987), it is not surprising that animals with accumbens shell lesions show reduced freezing and by implication, poorer retention when returned to the training context previously paired with an aversive footshock during initial conditioning (Jongen-Relo et al., 2003). Together, these studies suggest a functional relationship between the amygdala, ventral hippocampus and accumbens shell.

In light of the collective behavioral, neurochemical and anatomical evidence, Experiment 1 addressed whether memory enhancement produced by activating the BLA or HIPP following an emotionally arousing learning experience requires neuronal processing within the accumbens shell. In the present study, a surprising footshock was administered during training and followed by an intra-amygdala or hippocampal infusion of norepinephrine. If the accumbens shell contributes to this process, it is important to identify the timeframe that information initially encoded by the amygdala and hippocampus is further modified and processed within the accumbens shell. To address this issue, subjects received intra-amygdala or hippocampal infusion of norepinephrine at a dose previously shown to enhance memory (Hatfield and McGaugh, 1999; LaLumiere et al., 2003) and then given intra-accumbens infusion of a PBS control solution or muscimol to functionally inactivate the shell. Muscimol inactivation of the accumbens shell was delayed for 1 or 7 h to allow sufficient time for norepinephrine to activate intracellular cascades that lead to long-term synaptic modifications involved in forming new memories (Bergado et al., 2007; Alberini, 2009; Inda et al., 2011). If the accumbens mediates the consequences of limbic activation, then inactivation of the shell should attenuate memory for the aversive experience despite noradrenergic activation of either the hippocampus or amygdala.

In Experiment 2, a subset of the animals trained in the first study were exposed 48 h later to a generalization test in a Y-maze apparatus. The left and right allies were designed to have either similar or completely different contextual features as the original training with footshock in Experiment 1, and the latency to avoid the context with similar features was recorded. Findings from this study were expected to reveal if long term representations of the training context were differentially strengthened in the hippocampus relative to the amygdala by posttraining infusions of norepinephrine into each structure following footshock delivery in Experiment 1. If memories are strengthened by activating these limbic structures, then certain aspects of the memory trace should be evident when the same animals are given a generalization-test in a new apparatus constructed with similar and different contextual features as the original training environment.

GENERAL METHODS

All experimental procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the University of Virginia's Institutional Animal Care and Use Committee.

Subjects

Seventy-four male Sprague-Dawley rats (275–300 g) obtained from Charles River Laboratories (Wilmington, MA, United States) were used in these experiments. The rats were individually housed in polypropylene cages with corncob bedding and maintained on a standard 12:12 h light-dark cycle with lights on at 7:00 A.M. Food and water were available ad libitum during the 7 days adaptation period to the vivarium.

Surgery

Each rat received an injection of atropine sulfate (0.1 mg/kg i.p., American Pharmaceutical Partners, Inc., Schaumburg, IL, United States) and anesthetized with sodium pentobarbital (50 mg/kg, i.p., Abbot Laboratories, North Chicago, IL, United States). A midline scalp incision was made and 15 mm long extra thin wall stainless steel guide cannula (25 gauge, Small Parts, Miami Lakes, FL, United States) were implanted bilaterally in all subjects, 2 mm above the nucleus accumbens shell (AP +0.7, ML ± 1.0 from bregma, DV −5.4 from skull surface). Separate groups received additional bilateral cannula implants 2 mm above either the basolateral amygdala (AP −3.0, ML ± 5.0 from bregma, DV −6.7 from skull surface; $n = 36$) or the ventral subiculum of the hippocampus (AP −5.3, ML ± 4.5, DV −8.6 from skull surface; $n = 34$). All coordinates were adapted from the atlas of Paxinos and Watson (2005). Guide cannulae and two skull screws for anchoring were affixed to the skull with dental cement. The scalp was closed with sutures and stylets (15 mm, 00 insect dissection pins) were inserted into the four, implanted injection cannulae to prevent occlusion. Penicillin (0.1 ml i.m., Fort Dodge Animal Health, Fort Dodge, IA, United States) was administered immediately after surgery along with the analgesic, buprenex (0.05 ml s.c., Hospira, Inc., Lake Forrest, IL, United States). The rats remained in a temperature-controlled chamber for at least 1-h following surgery and were given 7 days to recover before initiating water deprivation procedures and behavioral training.

Histology

Rats were deeply anesthetized with a euthanasia solution and perfused intracardially with 0.9% saline followed by 10% formalin to verify microinjection cannulae placement. The brains were stored in a 10% formalin and 12% sucrose solution until sectioned on a vibratome. Sections were cut 60 μm thick, mounted on glass slides, subbed with chromium-aluminum and stained with cresyl violet. The location of the cannulae and injection needle tips were verified by examining enlarged projections of the slides. Data from animals with incorrect placements of guide cannula extending beyond the boundaries of the accumbens shell, basolateral amygdala complex or ventral hippocampus were excluded from statistical analysis. These criteria resulted in excluding the data of four animals from the BLA /Norepinephrine NAC-1hr muscimol, BLA/PBS NAC-1hr muscimol, HIPP /Norepinephrine NAC-1hr MUSC, and HIPP/PBS NAC-1hr PBS groups. A composite illustrating the location of injection needle tips within each of the three brain regions is shown in **Figure 1**.

METHODS FOR EXPERIMENT 1

Behavioral Paradigm: Water-Motivated Inhibitory Avoidance Task

Apparatus

A trough-shaped, two compartment rectangular apparatus (91 cm long, 21 cm wide at the top and 6.4 cm wide at the bottom) with a hinged lid was used to train the rats in a water-motivated inhibitory avoidance task. A sliding metal door (14.5 cm) separated a white and dark compartment. The white compartment was constructed of white opaque Plexiglas (31 cm long) and brightly illuminated by a 60 watt light located directly above the compartment. The dark compartment was constructed of stainless steel plates (60 cm long). A curved stainless steel water spout connected to a 30-cc plastic syringe containing water was placed 1 cm above the floor at the end of the dark compartment.

Training

One-week after surgery, rats were placed on a water restriction schedule with access to water for 20 min a day in addition to water consumed during behavioral training. Body weights were monitored daily to ensure weights did not deviate below 10% of their ad-lib feeding weights throughout the experiment. Animals were habituated by placing each in the inhibitory avoidance apparatus for 300 s to cross between the white and dark compartment and to explore the drinking spout.

For the next 6 days of training each rat was placed in the dark compartment facing the retractable door that separated the dark from the illuminated compartment. The metal door was lowered to 2/3 of its length (i.e., creating a 4 cm hurdle). A timer was started and the following measures were recorded until the completion of the trial: (a) latency to begin drinking, (b) total amount of time spent drinking, (c) total amount of time spent in the dark compartment and (d) total amount of time spent in the white illuminated compartment. Each training day consisted of one trial lasting 120 s.

On Day 7 (i.e., *experimental day*), each rat was placed in the dark compartment as before however, a 0.35 mA electrical footshock was administered manually as the rat initiated a lick toward the water spout. The shock remained on until the animal escaped the dark compartment by jumping over the 4 cm high hurdle into the illuminated white compartment. Each animal was retained in the white compartment for 30 s with the door two-thirds open. The door was then raised and animals were retained in the white compartment for an additional 30 s. Hence, the animals were allowed 60 s to learn that the white illuminated compartment was safe relative to the dark compartment where footshock was just experienced. Each animal was then removed from the apparatus and given intra-amygdala or hippocampal

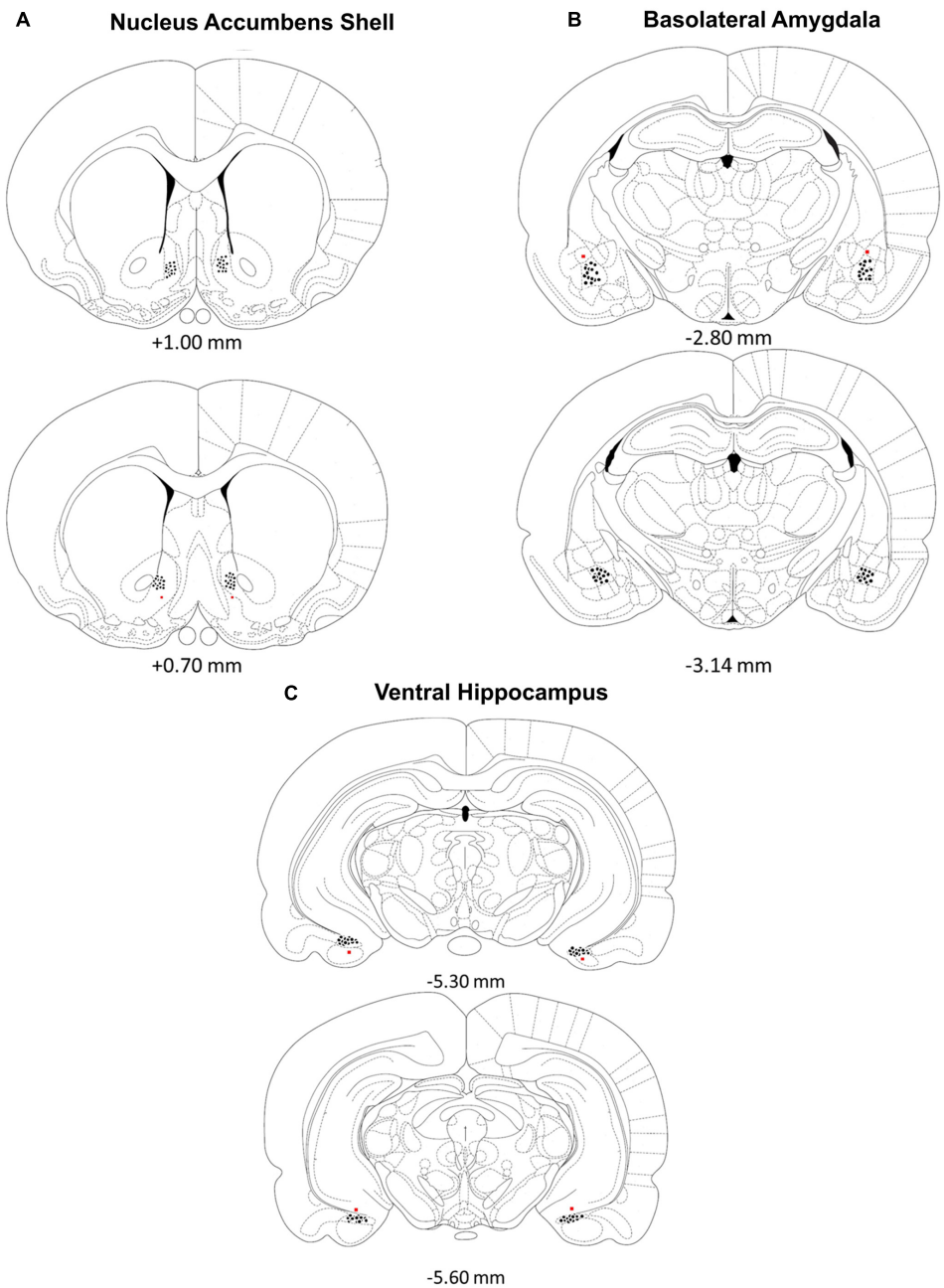

FIGURE 1 | (A–C) Location of injection needle tip placements in the **(A)** nucleus accumbens shell, **(B)** basolateral amygdala and **(C)** ventral hippocampus. Brain diagram from "The Rat Brain in Stereotaxic Coordinates"; adapted from Paxinos and Watson, 2005.

infusions of PBS or a dose of norepinephrine (0.2 μg/0.5 μl) previously shown to enhance memory (Izquierdo et al., 1992; Hatfield and McGaugh, 1999; LaLumiere et al., 2003). At either 1 or 7 h after the shock and amygdala or hippocampus injection, all subjects were removed from their home cages and given an intra-accumbens infusion of either muscimol (100 ng / 0.5 μl) or PBS. The dose of muscimol was based upon those that have been shown to impair memory in the accumbens shell (Scheel-Kruger et al., 1977; Reynolds and Berridge, 2002; Yang et al., 2005).

Microinjection Procedure

Each experimental rat was restrained by hand in the experimenter's lap, the stylets were removed and 17 mm, 30 gauge injection needles were inserted bilaterally into either the basolateral amygdala or ventral hippocampus. These injections were followed 1 or 7 h later by bilateral injections of PBS or muscimol (100 ng/0.5 μl) into the accumbens shell. The tip of the injection needles extended 2 mm beyond the base of the guide cannulae. The needles were connected to 10 μl Hamilton

syringes by PE-20 (polyethylene) tubing. An automated syringe pump (Sage-Orion, Boston, MA, United States) delivered 0.5 μl PBS, norepinephrine (0.2 μg; Sigma–Aldrich, St. Louis, MO, United States) or muscimol (100 ng; Sigma–Aldrich, St. Louis, MO, United States) over 60 s. The injection needles were left in place for an additional 60 s following infusions to ensure complete delivery of the drugs and the stylets were then reinserted into the cannulae. The number of subjects randomly assigned to the six basolateral amygdala (BLA) treatment groups is included in parenthesis *(1) BLA /PBS-NAC PBS 1hr = 5; (2) BLA /Noreipnephrine-NAC PBS 1 h = 7; (3) BLA /PBS-NAC Muscimol 1 h = 5; (4) BLA /Noreipnephrine-NAC Muscimol 1 h = 5; (5) BLA /PBS-NAC Muscimol 7 h = 5; (6) BLA /Noreipnephrine-NAC Muscimol 7 h = 5.*

The following subjects were included in the separate ventral hippocampus (HIPP) groups: *(1) HIPP/PBS-NAC PBS 1 h = 5; (2) HIPP /Noreipnephrine-NAC PBS 1 h = 5; (3) HIPP /PBS-NAC Muscimol 1 h = 5; (4) HIPP /Noreipnephrine-NAC Muscimol 1 h = 7; (5) HIPP /PBS-NAC Muscimol 7 h = 6; (6) HIPP/Noreipnephrine-NAC Muscimol 7 h = 6.*

Retention Test

Memory for the surprising footshock in the dark compartment was assessed 24 h later and consisted of two phases. During Phase 1, the rats were placed in the dark compartment facing the partially lowered metal door and given 60 s to enter the white compartment or alternatively, to initiate the first lick from the water spout. If the rat entered the white compartment, the metal door was raised and the rat remained in the white compartment for 30 s. Phase 2 of retention began after this 30-s period. Those that did not enter the white compartment after 60 s were removed and placed in the white compartment with the metal door raised for 30 s. Measures recorded during Phase 1 included latency to drink, amount of time spent drinking, and latency to escape into the white compartment. During Phase 2, the metal door was lowered and the time spent avoiding the dark compartment, latency to drink from the spout and total time spent drinking was recorded over 300 s.

Statistical Analysis

The behavioral measures from the water-motivated inhibitory avoidance task are expressed as mean ± standard errors (SE). Between-group comparisons for the behaviors measured during retention testing were made with a 2 × 3 analysis of variance (ANOVA) followed by *post hoc* tests.

METHODS FOR EXPERIMENT 2

Behavioral Paradigm: Y-Maze Task

Apparatus

In Experiment 2, a trough-shaped Y-maze constructed of stainless steel was used to examine the strength of the memory for the footshock experienced in Experiment 1 (**Figure 2**). The three alleys of the maze were each 49 cm long × 18.5 cm high. The floor and ceiling were 4 and 19 cm wide, respectively. The floor of the stem arm was covered by a removable cardboard panel embedded with tiny beads. This served as a neutral environment the animals had never experienced. The left and right alleys were constructed of two stainless steel plates separated lengthwise by a 0.5 cm gap, similar to the shock context in Experiment 1. However, an additional cardboard panel with fresh bedding was inserted into either the left or right arm in a counterbalanced fashion. This created two distinct arms; one that resembled a safe environment (fresh bedding normally used to line the rat's homecage) and one that resembled the context in which the animals had been previously shocked (steel plates). In addition, a water spout was positioned at the end of each alley to resemble the initial training environment. The bedding was changed after each animal and the apparatus was cleaned with a 10% ethanol solution.

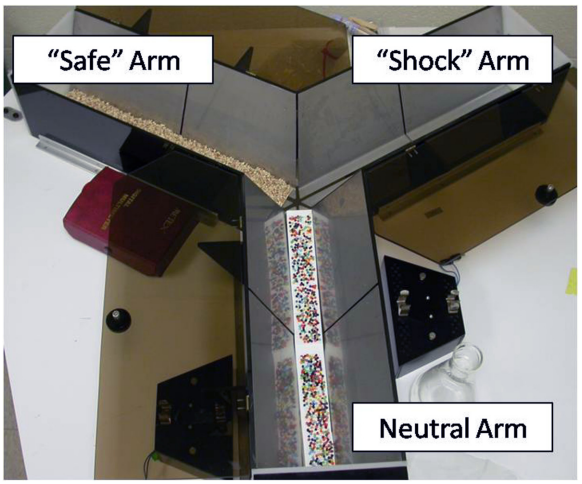

FIGURE 2 | Picture of the Y-maze used for the Generalization Test in Experiment 2. The neutral arm served as the main stem of the maze. Flooring for the neutral arm consisted of a beaded cardboard insert; a novel environment and texture. The left and right arms of the maze were counterbalanced so there was an equally likely chance of the right arm resembling the "shock" or "safe" arm. The "safe" arm consisted of fresh corncob bedding normally used to line the bottom of the animal's home cage. The "shock" arm had metal floors similar to those in the water-motivated inhibitory avoidance task in which animals were previously shocked.

Generalization Testing

Forty-eight hours following footshock in the water-motivated inhibitory avoidance task of Experiment 1, a subset of animals were tested in the Y-Maze task. Specifically, only those animals that received either PBS or norepinephrine injections in the basolateral amygdala or hippocampus *and* PBS or muscimol 1 h later in the accumbens shell. Only the 1 h muscimol treatment groups were tested in the Generalization Test since there were no statistically significant differences between the 1 h and 7 h musicmol groups on any of the behavioral measures recorded in Experiment 1. Testing began by placing an animal in the neutral arm of the Y-maze. Each animal was allowed 300 s to explore the maze. The location of the "safe" and "shock" arms were counterbalanced to eliminate possible confounds that may evolve from left or right biases. Measurements included (1) latency to enter the "shock" arm, (2) latency to lick from the spout in the

shock arm and (3) cumulative time spent drinking from the water spout.

Statistical Analysis

The behavioral measures from the Y-maze task are expressed as mean ± standard errors (SE). Between-group comparisons for the behaviors measured during retention testing were made with a 2 × 2 × 2 analysis of variance (ANOVA) followed by *post hoc* tests.

RESULTS FOR EXPERIMENT 1

Basolateral Amygdala Injections

Phase 1 Retention Testing

First lick latency in the shock compartment

A 2 × 2 ANOVA on mean latency to first lick during Phase 1 of retention testing did not differ statistically between the separate control and treatment groups, $F(2,30) = 1.27$, $p > 0.05$. Subjects from each group displayed a lick response during Phase 1 and the mean latency for this response ranged from 25.3 to 51.2 s. The means for each group given basolateral injections followed 1 or 7 h later by infusions into the accumbens shell are: PBS/1 h PBS $M = 29.32$, PBS/1 h Muscimol $M = 35.52$, PBS/7 h Muscimol $M = 39.86$, NE/1 h PBS $M = 39.39$, NE/1 h Muscimol $M = 25.36$, and NE/7 h Muscimol $M = 51.20$).

Escape latency from the shock compartment

A 2 × 2 ANOVA on mean latency to exit the shock context and enter the white compartment during the 1-min retention test revealed no significant interaction between the treatment groups, $F(2, 30) = 1.34$, $p > 0.05$. All animals remained in the shock compartment without crossing into the illuminated area of the apparatus for a similar amount of time. The mean escape latency and number of rats per group that remained in the dark compartment for the 60 s. retention test are included in parenthesis. (PBS/1 h PBS 60.0 ± 0.0, $n = 5$; PBS/1 h Muscimol 60.0 ± 0.0, $n = 5$; PBS/7 h Muscimol 52.9 ± 15.7, $n = 4$; NE/1 h PBS 46.6 ± 23.9, $n = 6$; NE/1 h Muscimol 60.0 ± 0.0, $n = 5$; and NE/7 h Muscimol 56.4 ± 9.4, $n = 4$).

Phase 2 Retention Testing

Contextual memory

The second phase of retention testing began by placing each subject in the white compartment of the apparatus facing away from the retractable door. After a 30 s delay, access to the dark (shock) compartment was made possible by lowering a guillotine door that separates the two sections of the apparatus. Latency to enter the dark compartment was then recorded and served as an index of memory for the footshock experienced 24 h earlier. A 2 × 2 ANOVA indicated no significant interaction between basolateral amygdala (BLA) and accumbens treatments for the latency to enter the shock compartment, $F(2,30) = 1.00$, $p > 0.05$. As shown in **Figure 3A**, all experimental groups spent a similar length of time in the white compartment before entering the context where footshock was administered 24 h previously.

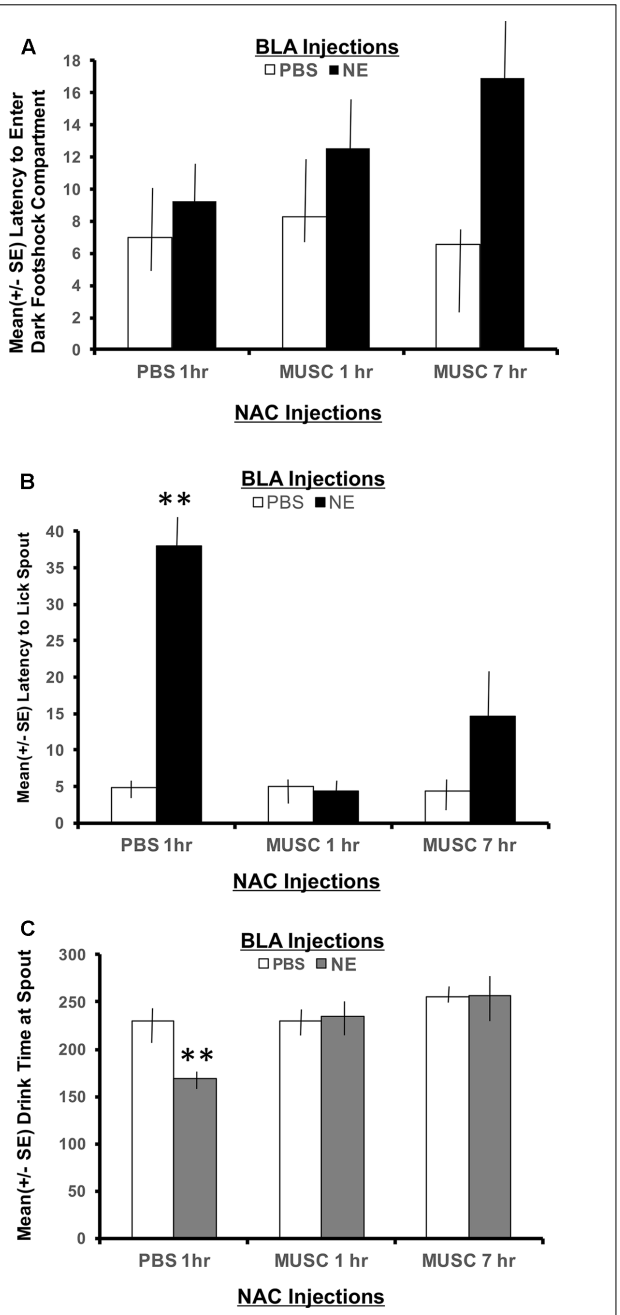

FIGURE 3 | (A) Mean (+SE) latency for subjects with bilateral basolateral and accumbens shell cannulae implants to enter the compartment where shock was delivered 24 h earlier. There were no group differences in the time it took animals to enter the shock context. A 2 × 2 ANOVA revealed no significant differences between PBS controls and all other treatment groups to enter the dark, footshock compartment of the apparatus. **(B)** Mean (+SE) latency for animals with basolateral amygdala and accumbens shell cannulae implants to lick the spout after entering the shock compartment. Although all groups readily entered the dark compartment, only animals given norepinephrine (NE) in the basolateral amygdala (BLA) and PBS in the accumbens (NAC) took significantly longer to traverse the dark compartment and initiate licking from the spout (**$p < 0.01$). The enhancement produced by BLA infusion of NE was blocked by intra-accumbens suppression of neuronal activity with Muscimol either 1 or 7 h following the NE injections. ** denotes $p < 0.01$.

(Continued)

FIGURE 3 | Continued
(C) Mean (+SE) time spent drinking from the water spout in the footshock compartment in animals with basolateral and accumbens shell cannulae implants. The cumulative time spent drinking was significantly reduced in animals given intra-basolateral infusion of norepinephrine and intra-accumbens PBS ($p < 0.01$). Inactivation of the accumbens shell with muscimol 1 or 7 h later blocked this effect. Animals in the NE/1 h MUSC and NE/7 h MUSC groups drank for a similar amount of time as non-shock control animals. ** denotes $p < 0.01$.

Latency to first lick

The footshock administered on day 7 of training was not delivered until the subject approached the spout and initiated its first lick to drink. As such, the representations of this event that may be encoded into memory include, (1) the context in which the footshock occurred and (2) the instrumental action emitted before delivery of the footshock (approaching the spout to drink). To assess memory for the second possible representation, the time required to drink from the spout after entering the shock context as well as the duration of time spent drinking was recorded. As shown in **Figure 3B**, a 2 × 2 ANOVA revealed a significant interaction between BLA and accumbens treatments, $F(2,30) = 4.55$, $p < 0.05$. Each of the treatment groups readily entered the dark shock context containing the water spout at the beginning of Phase 2 testing. However, subjects in the group given posttraining infusion of norepinephrine in the BLA and PBS 1 h later in the accumbens shell (NE/1 h PBS) took significantly more time to initiate drinking from the water spout relative to all other treatment groups ($p < 0.01$).

The memory enhancing actions of norepinephrine on BLA functioning was attenuated by suppressing accumbens neuronal activity with muscimol. The lick latencies of subjects given muscimol into the accumbens either 1 or 7 h following the norepinephrine treatment were indistinguishable from those of PBS controls ($p > 0.05$) and significantly shorter than subjects given the same dose of norepinephrine in the BLA (NE/1 h PBS compared to NE/1 h Muscimol, $p < 0.01$ and NE/7 h Muscimol, $p < 0.01$).

Time spent drinking

The duration of time spent consuming water from the spout after initial contact was made was also recorded. This measure served as an additional index of memory for the lick-footshock association developed after the Day 7 training. A 2 × 2 ANOVA revealed a significant interaction between treatment groups for the mean time spent drinking from the spout where footshock occurred 24 h earlier, $F(2,30) = 4.60$, $p < 0.05$ (**Figure 3C**). As assessed by *post hoc t*-tests, animals in the NE/1 h PBS group spent significantly less time drinking from the spout relative to all other treatment groups ($p < 0.01$ for PBS/1 h PBS, PBS/1 h Muscimol, PBS/7 h Muscimol, NE/1 h Muscimol and NE/7 h Muscimol). Again, regardless of the time delay between BLA and accumbens injections (1 h vs. 7 h), muscimol in the accumbens blocked the influence of activating noradrenergic receptors in the basolateral amygdala. Muscimol given 1 or 7 h later in PBS animals, however had no effect on performance as compared to PBS/1 h PBS animals ($p > 0.05$ for PBS/1 h Muscimol and PBS/7 h Muscimol).

Hippocampus Injections

Phase 1 Retention Testing

First lick latency in the shock compartment

A 2 × 2 ANOVA on mean latency to first lick during Phase 1 of retention testing showed a significant difference between the treatment groups, $F(2,28) = 3.66$, $p < 0.05$. *Post hoc* comparisons revealed that the group given post training ventral hippocampal infusion of norepinephrine and accumbens PBS refrained from licking the spout significantly longer than all other groups during this first phase of testing (PBS/1 h PBS $M = 35.88$, PBS/1 h Muscimol $M = 30.12$, PBS/7 h Muscimol $M = 52.83$, NE/1 h PBS $M = 60.00$, NE/1 h Muscimol $M = 37.36$, and NE/7 h Muscimol $M = 38.47$). The remaining control and treatment groups displayed similar latencies to lick the spout during this initial test.

Latency to escape the shock compartment

Similar to the findings obtained with the basolateral amygdala groups, the mean latency to exit the shock context and enter the white compartment was not statistically different between the separate hippocampal treatment groups as assessed by a 2 × 2 ANOVA (HIPP × accumbens treatment), $F(2,28) = 2.22$ $p > 0.05$ (data not shown). All experimental groups remained in the shock compartment for a similar amount of time during this initial phase of retention testing. The mean escape latency and number of rats per group that remained in the dark compartment for the 60 s retention test are included in parenthesis. (PBS/1 h PBS 56.0 ± 8.9, $n = 4$; PBS/1 h Muscimol 60.0 ± 0.0, $n = 5$; PBS/7 h Muscimol 41.2 ± 21.4, $n = 3$; NE/1 h PBS 55.0 ± 11.2, $n = 4$; NE/1 h Muscimol 49.8 ± 15.2, $n = 5$; and NE/7 h Muscimol 54.7 ± 13.0, $n = 4$).

Phase 2 Retention Testing

Contextual memory

To measure memory for the context where footshock was delivered, the latency to enter the shock compartment was measured from the beginning of Phase 2 (animals start in the white compartment). As shown in **Figure 4A**, a 2 × 2 ANOVA revealed a significant interaction between hippocampal (HIPP) and accumbens infusions, $F(2,28) = 4.22$, $p < 0.05$. All treatment groups preformed in a similar fashion, except the NE/1 h PBS group. Animals given norepinephrine in the hippocampus and PBS in the accumbens an hour later, took significantly longer to exit the white "safe" compartment and enter the dark context where footshock was delivered 24 h earlier ($p < 0.01$ compared to all treatment groups). Infusion of muscimol in the accumbens either 1 or 7 h later attenuated the memory enhancing effect of activating the hippocampus with norepinephrine ($p < 0.01$ for NE/1 h Muscimol and NE/7 h Muscimol groups compared to NE/1 h PBS group).

Latency to first lick

A 2 × 2 ANOVA also revealed significant differences between treatment groups on the latency to lick the spout after entering the dark compartment, $F(2,28) = 10.80$, $p < 0.01$. The NE/1 h

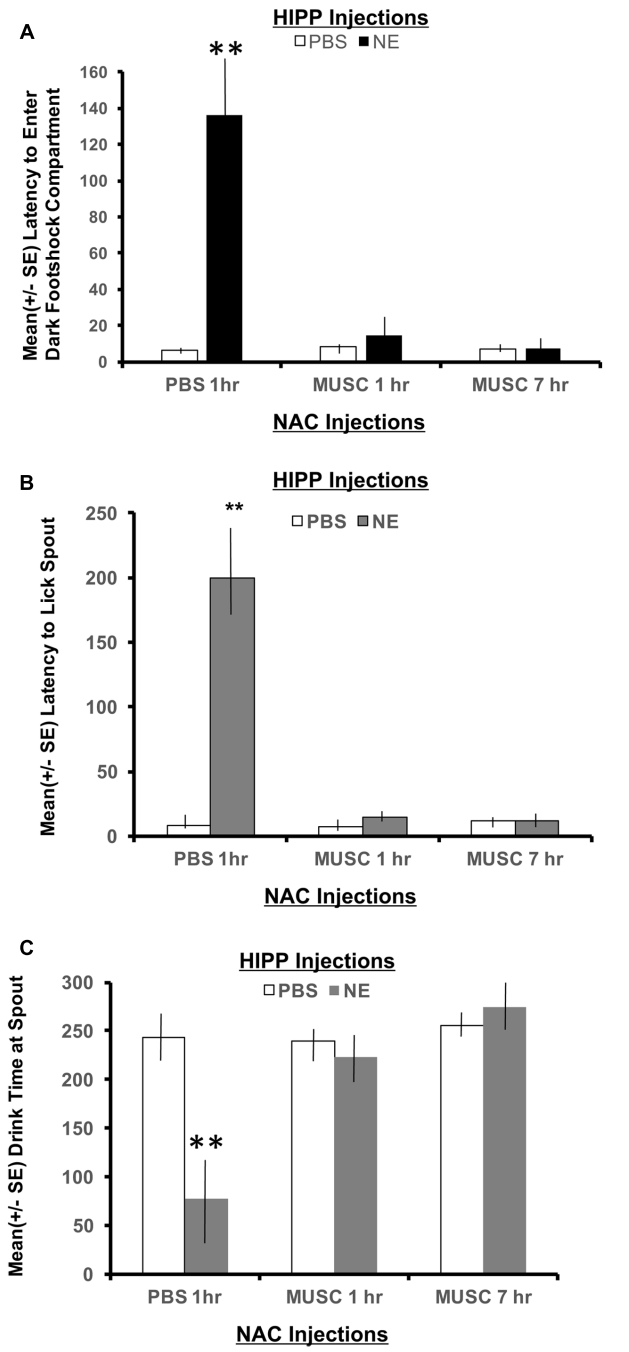

FIGURE 4 | (A) Mean (+ SE) latency to enter the compartment where shock was administered 24 h previously in animals with ventral hippocampal and accumbens shell cannulae implants. Only animals given norepinephrine in the hippocampus took significantly longer to enter the context where footshock had been delivered 24 h previously ($p < 0.01$). Infusion of muscimol in the accumbens either 1 or 7 h later attenuated the memory enhancing effect ($p < 0.01$ for NE/1 h MUSC and NE/7 h MUSC groups compared to NE/PBS group). These findings are in direct contrast to those depicted in **Figure 3A** for animals given the same dose of norepinephrine in the basolateral amygdala. ** denotes $p < 0.01$. **(B)** Mean (+SE) latency for animals with ventral hippocampal and accumbens shell cannulae implants to initiate licking from the spout after entering the dark compartment. Not only did animals in

(Continued)

FIGURE 4 | Continued
the NE/1 h PBS group take longer to enter the shock context, but they also took significantly longer to initiate drinking from the spout compared to all other treatment groups (**$p < 0.01$). **(C)** Mean (+SE) time spent drinking from the water spout located in the dark compartment on a retention test given 48 h after training with a footshock. Disruption of accumbens neuronal functioning via infusions of muscimol either 1 or 7 h later blocks the memory enhancement of activating ventral hippocampal neurons with norepinephrine. This effect is illustrated by the length of time these groups spent drinking compared to animals treated with the same dose of norepinephrine in the hippocampus and PBS in the accumbens. Only NE/1 h PBS animals spent a significantly less amount of time drinking from the water spout (**$p < 0.01$).

PBS group took significantly longer to enter the shock context than any other group and also required a significantly longer period of time to lick the spout ($p < 0.01$, compared to all treatment groups; **Figure 4B**). Muscimol infusions into the accumbens shell that were delayed either 1 or 7 h posttraining blocked the effects of noradrenergic activation of the ventral hippocampus and attenuated the extended time to both enter the footshock compartment and initiate licking from the water spout.

Time spent drinking

There was also a significant interaction between treatment groups on the cumulative time spent drinking from the spout as revealed by a two-way ANOVA, $F(2,28) = 8.54$, $p < 0.01$ (**Figure 4C**). *Post hoc t*-tests revealed that animals in the NE/1 h PBS group spent significantly less time drinking from the spout relative to all other treatment groups ($p < 0.01$ for PBS/1 h PBS, PBS/1 h Muscimol, PBS/7 h Muscimol, NE/1 h Muscimol and NE/7 h Muscimol). Despite the time delay between HIPP and accumbens injections (1 h vs. 7 h), muscimol in the accumbens blocked the influence of activating noradrenergic receptors in the basolateral amygdala. Muscimol given 1 or 7 h later in PBS animals, however had no effect on performance as compared to PBS/1 h PBS animals ($p > 0.05$ for PBS/1 h Muscimol and PBS/7 h Muscimol).

Comparison between BLA and HIPP Treatments

Since all groups in Experiment 1 experienced identical training and footshock procedures, it was possible to determine whether posttraining treatments rendered within the basolateral amygdala versus the hippocampus differentially affects memory for the separate responses that are measured during retention testing. The water-motivated inhibitory avoidance task has been used to assess the contribution of amygdala processing during arousing situations (Miyashita and Williams, 2002). The task design also has strong contextual features that require hippocampal processing. Thus, an assessment of performance in groups given PBS or NE within these structures and only PBS in the accumbens shell should determine which aspects of this task are more sensitive to amygdala versus hippocampal processing.

A 2×2 ANOVA (amygdala or hippocampus × norepinephrine or PBS) revealed significant differences between groups for two critical measures. Animals given PBS in either the basolateral amygdala or ventral hippocampus perform in a similar fashion across measures of latency to enter the shock compartment

and latency to initiate licking from the spout after entering the dark compartment. However, there are significant differences in basolateral amygdala norepinephrine infusions compared with hippocampal norepinephrine infusions. Animals given norepinephrine in the hippocampus took significantly longer to enter the shock compartment, $F(1,18) = 4.33$, $p < 0.05$, and drink from the water spout, $F(1,18) = 7.47$, $p < 0.05$, relative to the basolateral groups given the same treatment (*data not shown*).

RESULTS FOR EXPERIMENT 2

A second experiment was conducted to assess the strength of the representation of footshock training retained in memory for the norepinephrine and muscimol treatment groups (both BLA and hippocampal) used in Experiment 1. For this purpose, only subjects given amygdala or hippocampal PBS or norepinephrine followed by accumbens treatment of 1 h PBS or 1 h muscimol during Experiment 1 were given a generalization test in a Y-maze apparatus. Two basolateral and two hippocampus animals were unable to complete this task and were excluded from data analysis. The Y-maze apparatus was modified such that only one of the maze alleys contained the same contextual attributes (i.e., metal walls and footshock plates) that were present during footshock delivery in Experiment 1 whereas the remaining two alleys were constructed with completely different contextual features.

Basolateral Amygdala versus Hippocampus

Using a 2 × 2 × 2 ANOVA (BLA/HIPPx PBS/NE x NAC PBS/NAC Muscimol 1 h), it is possible to determine if animals given amygdala or hippocampus treatments perform differently in this task designed to assess the strength of the footshock representation in Experiment 1. The ANOVAs revealed a significant interaction between amygdala, hippocampal and accumbens treatments on the latency to enter the shock resembling arm $F(1,32) = 6.01$, $p < 0.05$ as well as the latency to first lick from the spout in the arm that resembled the shock compartment, $F(1,32) = 8.38$, $p < 0.01$. As shown in **Figure 5A**, only animals with hippocampal infusions of norepinephrine and intra-accumbens PBS took significantly longer to enter the shock arm ($p < 0.01$ compared to all BLA and HIPP treatment groups). These animals also took significantly more time to initiate licking from the spout located in the "shock" arm ($p < 0.01$ compared to all BLA and HIPP treatment groups; **Figure 5B**). Disruption of accumbens processing 48 h previously with muscimol attenuated these two effects within hippocampus treated animals. Infusions of norepinephrine in the basolateral amygdala did not produce any appreciable differences in performance as compared to PBS/PBS controls.

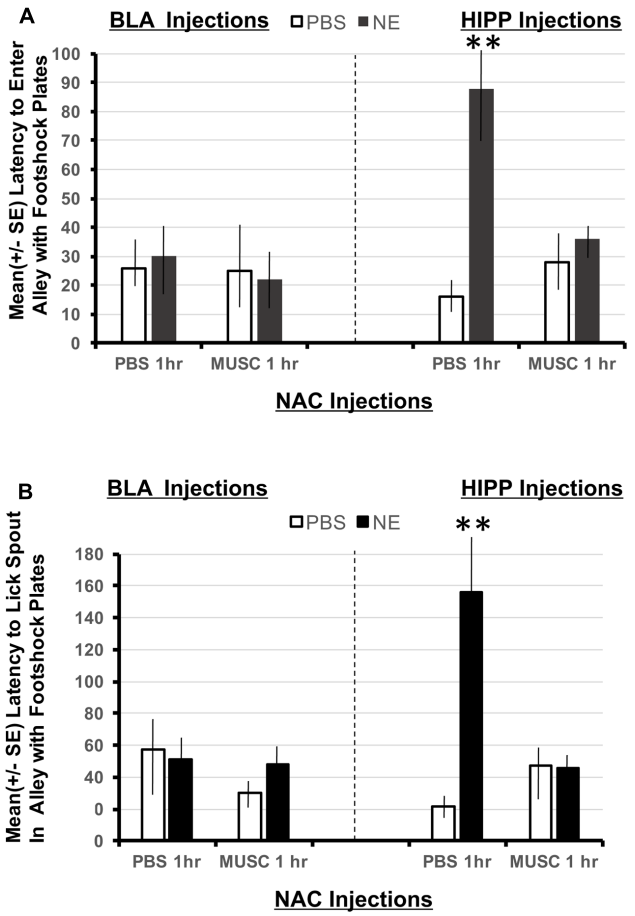

FIGURE 5 | (A) Mean (+SE) latency to enter the Y-maze alley resembling the footshock context in animals with accumbens shell cannulae implants and implants in either the basolateral amygdala or ventral hippocampus. Only animals given norepinephrine (NE) in the hippocampus took significantly longer to enter the arm of the Y-maze that resembled the footshock context ($p < 0.01$). Although the shock and injection was given 48 h previously, these animals maintain high contextual memory for the shock compartment. This effect is blocked in animals given muscimol in the accumbens. ** denotes $p < 0.01$. **(B)** Mean (+SE) latency to lick from the water spout located in the Y-maze ally resembling the footshock context in animals with accumbens shell cannulae implants and implants in either the basolateral amygdala or ventral hippocampus. Animals in the BLA-NE/PBS not only readily entered the similar shock context, but they also readily drank from the spout located at the end of the arm. However, the same dose of norepinephrine in the hippocampus produced significantly longer latencies to lick from the spout compared to all other treatment groups ($p < 0.01$).

DISCUSSION

Although findings from anatomical and physiological studies revealed that amygdala and hippocampal inputs converge on single neurons in the accumbens shell (French and Totterdell, 2003), evidence suggesting that accumbens processing is necessary to integrate new learning experiences from these limbic areas into memory is scarce. Moreover, experimental findings implicating the actual time frame in which the accumbens contributes to encoding novel events into memory storage has not been successfully documented. Results emerging from the present experiments are instrumental in addressing both of these shortcomings in the literature.

Findings from Experiment 1 confirm previous studies demonstrating that posttraining activation of noradrenergic receptors within the basolateral amygdala or hippocampus facilitate subsequent retention performance (Bevilaqua et al., 1997; Izumi and Zorumski, 1999; Birthelmer et al., 2003; Miranda et al., 2003; van Stegeren et al., 2005, 2008; Roozendaal et al., 2006, 2008; Tully et al., 2007; Dommett et al., 2008). The present results also extend these findings by revealing that noradrenergic activation of the amygdala or hippocampus differentially facilitates the category of representations formed after emotionally arousing events involving unexpected footshock. For example, noradrenergic activation of the amygdala facilitates memory for response specific representations directly associated with footshock delivery (**Figure 3B**), whereas activation of the hippocampus enhances memory for the context in which the emotionally arousing footshock is delivered (**Figure 4A**).

Second, animals given intra-hippocampal infusions of norepinephrine (HIPP-NE) took significantly longer than all other treatment groups to enter the Y-maze alley in Experiment 2 that was contextually similar to the dark compartment where footshock was delivered in the first study. This finding indicates that noradrenergic activation of the hippocampus not only leads to more stable representations of the footshock event over time, but this memory also generalizes to new learning conditions involving similar contextual stimuli. In contrast, the response specific memory associated with licking the spout that was evident in subjects given intra-amygdala infusions of norepinephrine was not as robust when this group was placed in the Y-maze although it contained similar contextual features. These animals readily entered the context of the Y-maze containing the metal footshock plates and did not hesitate before drinking from the water spout (**Figures 5A,B**).

The most intriguing findings of the current study reveal that the consequences of activating noradrenergic receptors in the amygdala or hippocampus are mediated in part by actions initiated within the accumbens shell. The data show that inactivation of the shell with the GABAergic agonist muscimol, attenuates memory enhancement produced by activating either the amygdala or hippocampus. This attenuation in memory was evident when neuronal activity in the accumbens shell as interrupted either 1 or 7 h after the limbic drug infusions. These results extend what is currently known regarding the time frame in which the accumbens contributes to mnemonic processing (Lorenzini et al., 1995) and demonstrates that this activity is critical during the initial and late stages of consolidation.

Differences in Amygdalar and Hippocampal Processing

Results from electron microscopy studies confirm that both the amygdala and hippocampus converge on single output neurons in the accumbens shell (French and Totterdell, 2003). Based on this anatomical arrangement, accumbens neurons are in an ideal position to integrate representations of new learning experiences that are initially processed by the amygdala and hippocampus. In support of this view, Roozendaal et al. (2001) found that post-training infusions of compounds that facilitate retention when given in either the amygdala or hippocampus, were ineffective in influencing memory in animals given pre-training accumbens lesions. Other manipulations that interrupt normal accumbens synaptic activity such as microinfusions of tetrodotoxin also impair retention of a footshock given in a similar inhibitory avoidance task even when the injections are delayed for 90 min following training (Lorenzini et al., 1995). Together, these results suggest that processing in the hippocampus or amygdala alone may not be sufficient to influence memory, but may require additional integration within the accumbens.

Results from the current study are in concordance with previous findings (Bevilaqua et al., 1997) that noradrenergic activation of the amygdala or hippocampus facilitates memory for an arousing footshock experience. Using a one-trial step down inhibitory avoidance task, Bevilaqua et al. (1997) found that basolateral activation enhances memory only when norepinephrine is administered immediately post-training. Noradrenergic activation of the hippocampus, however, enhances memory for the footshock experience when administered 0, 3, or 6 h post-training. These data suggest that memory modulation in the hippocampus occurs for up to 6 h compared to amygdala modulation. However, measures of step-down latency used in the previous study fail to discern the specific contributions each limbic structure provides to the memory representation of the footshock experience. For example, information conveyed from the basolateral amygdala to the accumbens shell plays a crucial role in the learning and storing into memory the motivational value of stimuli. In contrast, hippocampal afferents to the accumbens provide information regarding contextual features of the environment. Results from the present work not only show that noradrenergic activation of limbic structures enhances memory, but reveal key differences in activating the amygdala or hippocampus.

The behavioral paradigm used in Experiment 1, was developed to dissociate representations in memory for the contextual versus the response specific aspects of learning that occur following unexpected footshock delivery. Therefore, it was possible to evaluate the differential contributions each limbic structure provides following activation. Results show that activation of the basolateral amygdala had no effect on the latency to enter the context where footshock had been administered 24 h previously (**Figure 3B**). However, this treatment was shown to facilitate memory on measures relating to response specific aspects of the task such as latency to lick the spout and the cumulative time spent drinking (**Figures 3B,C**). In contrast to amygdala activation, infusions of norepinephrine in the ventral hippocampus facilitate memory for the context in which the footshock transpired (**Figure 4A**). Because these animals took significantly longer to enter the shock context, they also have longer latencies to initiate drinking from the water spout. These behavioral findings provide functional evidence that supports electrophysiological data showing that hippocampal activation generates longer durations of accumbens activity as compared to amygdala stimulation (Grace, 2000). Together with the current behavioral data, it can be suggested that the longer periods of neuronal activity in the accumbens in response to hippocampal activation reflect the attention required to process contextual

cues. During initial training in the water-motivated inhibitory avoidance task, animals learn that a context that was once pleasant (provided the availability of water) is now aversive (footshock). On the other hand, it can be suggested that the brief period of accumbens neuronal activity following amygdala stimulation reflects event-related processing. This is supported by findings that, although animals infused with norepinephrine in the amygdala readily enter the shock compartment, they still require a significantly longer period of time to begin drinking from the spout (last response emitted before the footshock was delivered). The difference in the magnitude of memory enhancement between basolateral and hippocampal animals shown in the current study provide behavioral support for the view that during emotionally salient events, hippocampal input may dominate with contextual processing compared to basolateral amygdala input, which may tag the affective value of the situation (Grace, 2000; Gill and Grace, 2011).

In contrast to the current results, a separate study found the basolateral amygdala to be involved in contextual learning and that processing in the hippocampus is not required 6 h post-training (Sacchetti et al., 1999). Several procedural dissimilarities underlie the discrepancy reported between these data and the current findings. First, it should be noted that Sacchetti et al. (1999) used Pavlovian fear conditioning procedures such that a tone preceded a footshock. The pairing of tone with a shock occurred seven times, giving the animal ample time to associate the tone and shock with the context. In the current study, animals were given only a single footshock that did not persist beyond 2 s before they escaped into the white compartment. An additional difference between the two behavioral paradigms is that the current study required an instrumental response of approaching the spout before the footshock was delivered. This allows for a more precise association between action and stimulus (shock). A third discrepancy that should be noted is the target area of the hippocampus. Sacchetti et al. (1999) found that processing in the dorsal hippocampus is not required 6 h after the footshock experience. This means that 6 h post-training, blockade of dorsal hippocampal neurons has no influence on memory. But the current study investigated the contribution of ventral hippocampal processing to memory consolidation. If the ventral hippocampus were similar to the dorsal, then only animals given norepinephrine in the hippocampus and muscimol in the accumbens1 h later would show attenuation in contextual memory. However, the current results showed that an intact pathway from ventral hippocampus to accumbens is required 7 h post-training in order to facilitate memory for where the footshock occurred. Taken together, these findings suggest that dorsal and ventral hippocampal areas not only differ in the pattern of innervation to the accumbens, but also in the length of time that neuronal processing is required to facilitate contextual and spatial memories.

Gating of Limbic Information within the Nucleus Accumbens Shell

Several studies indicate that accumbens neurons require activation from more than one source to reach threshold (DeFrance et al., 1985; Callaway et al., 1991). This constraint on activity may explain why accumbens neurons receive inputs from several memory related areas. In particular, projections from the basolateral amygdala and ventral hippocampus converge monosynaptically on projection neurons within the caudomedial region of the accumbens shell (French and Totterdell, 2003; Britt et al., 2012; Correia et al., 2016). Because the accumbens receives converging inputs from multiple areas, it is important to understand how separate inputs may regulate neuronal firing in this structure. An emerging idea in the literature dealing with the functionality of the nucleus accumbens is the hypothesis of neural networks. Pennartz et al. (1994) propose that the accumbens is comprised of neuronal ensembles. These ensembles are best understood in terms of their collective activation.

McGinty and Grace (2009) demonstrated that nucleus accumbens neurons integrate limbic and cortical innervations depending on the intensity and timing of inputs. Specifically, weak stimulation of two inputs generates more excitation of accumbens neurons than activation of either structure alone. When these stimulations occur at the same time, accumbens neurons become active. This electrophysiological characteristic of neurons in the accumbens establishes a coincidence detection system such that areas that fire together have direct influence over accumbens activity. However, an interesting finding by McGinty and Grace (2009) showed that strong activation of one input may disrupt processing of the second input. This electrophysiological feature may serve as the mechanism underlying behavioral differences in hippocampal and amygdala activation reported in the present study. Although activation of both limbic structures facilitates memory for certain aspects of the water-motivated inhibitory avoidance task, the magnitude of the facilitation was greater in subjects receiving post-training noradrenergic activation of the ventral hippocampus. The reason may be due to the fact that delivery of the footshock in Experiment 1 continued the whole length of the dark compartment until animals escaped into the safe/white compartment. The animals remained in the white compartment for 30 s before the retractable door was raised. During this 30 s period of time, animals could see the dark compartment and form a distinct representation between the "dark" shock and "illuminated" safe compartments of the apparatus. This component of the training procedure may account for the stronger degree of activation in the hippocampus. This strong activity may have disrupted amygdala processing as proposed by McGinty and Grace (2009), leading to a facilitation in contextual measures in animals with norepinephrine infusions in the hippocampus as compared to the amygdala.

Significance of Accumbens Involvement in Long-Term Consolidation

The accumbens not only plays a role in the integration of information emanating from the hippocampus or amygdala, but is also involved in the consolidation of these processes into memory. For example, rats with accumbens lesions fail to modify response latencies or reaction time to cues that lead to aversive outcomes, such as the delivery of quinine in the place of an anticipated liquid sucrose reward

(Schoenbaum and Setlow, 2003). The consolidation of memory for these and other types of emotionally arousing events is a time dependent process that does not happen instantly, but rather occurs over hours. Studies employing functional inactivation techniques to produce reversible lesions demonstrate that neuronal activity in the accumbens is crucial for consolidation for at least 90 min following learning (Lorenzini et al., 1995).

While findings from the current study are in agreement with previous results (Lorenzini et al., 1995), they also extend what is currently known about the integrative nature of accumbens neurons and the timeframe in which accumbens processing is required. First, the present work demonstrates that activation of the amygdala or hippocampus is not sufficient to enhance memory when accumbens activity is disrupted with muscimol. Second, results from the present study determine the temporal window in which neurons from the accumbens are required to process the beneficial information emanating from the amygdala or hippocampus. Findings show that without accumbens processing 1 or 7 h post-training, memory for a footshock experience is attenuated despite limbic activation. This timeframe corresponds to phases of synaptic plasticity documented in the hippocampus up to 6 h post-training with microarray analysis (O'Sullivan et al., 2007).

CONCLUSION

This study provides evidence that blocking accumbens functioning with muscimol an hour or even 7 h following amygdala or hippocampus activation attenuates the improvement in memory seen following noradrenergic activation of the amygdala or hippocampus alone. These findings suggest that the accumbens shell plays an integral role modulating information initially processed by limbic structures following exposure to emotionally arousing events. Additionally, results are integral in determining the involvement of the accumbens shell in long-term consolidation processes lasting over 6 h.

AUTHOR CONTRIBUTIONS

The experiments were designed in collaboration between EK and CW. EK conducted the studies and analyzed the data. The final manuscript was written together by both authors.

FUNDING

Research supported by The National Science Foundation (0720170 to CW).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 12 February 2018
doi: 10.3389/fphar.2018.00042

Chronic MK-801 Application in Adolescence and Early Adulthood: A Spatial Working Memory Deficit in Adult Long-Evans Rats But No Changes in the Hippocampal NMDA Receptor Subunits

Libor Uttl[1,2], Tomas Petrasek[3], Hilal Sengul[3,4], Marketa Svojanovska[3], Veronika Lobellova[3], Karel Vales[2,3], Dominika Radostova[3,5], Grygoriy Tsenov[1], Hana Kubova[1], Anna Mikulecka[1], Jan Svoboda[3] and Ales Stuchlik[3]**

[1] Department of Developmental Epileptology, Institute of Physiology, Czech Academy of Sciences, Prague, Czechia, [2] Department of Experimental Neurobiology, National Institute of Mental Health, Klecany, Czechia, [3] Department of Neurophysiology of Memory, Institute of Physiology, Czech Academy of Sciences, Prague, Czechia, [4] Radboud Institute for Molecular Life Sciences, Radboud University, Nijmegen, Netherlands, [5] Second Faculty of Medicine, Charles University, Prague, Czechia

The role of NMDA receptors in learning, memory and hippocampal function has long been recognized. Post-mortem studies have indicated that the expression or subunit composition of the NMDA glutamate receptor subtype might be related to the impaired cognitive functions found in schizophrenia patients. NMDA receptor antagonists have been used to develop animal models of this disorder. There is accumulating evidence showing that not only the acute but also the chronic application of NMDA receptor antagonists may induce schizophrenia-like alterations in behavior and brain functions. However, limited evidence is available regarding the consequences of NMDA receptor blockage during periods of adolescence and early adulthood. This study tested the hypothesis that a 2-week treatment of male Long-Evans and Wistar rats with dizocilpine (MK-801; 0.5 mg/kg daily) starting at postnatal days (PD) 30 and 60 would cause a long-term cognitive deficit and changes in the levels of NMDA receptor subunits. The working memory version of the Morris water maze (MWM) and active place avoidance with reversal on a rotating arena (Carousel) requiring cognitive coordination and flexibility probed cognitive functions and an elevated-plus maze (EPM) was used to measure anxiety-like behavior. The western blot method was used to determine changes in NMDA receptor subunit levels in the hippocampus. Our results showed no significant changes in behaviors in Wistar rats. Slightly elevated anxiety-like behavior was observed in the EPM in Long-Evans rats with the onset of treatment on PD 30. Furthermore, Long-Evans rats treated from PD 60 displayed impaired working memory in the MWM. There were; however, no significant changes in the levels of NMDA receptor subunits because of MK-801 administration. These findings suggest that a 2-week treatment starting on

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Edited by:
Alfredo Meneses, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Mexico

Reviewed by:
Tomiki Sumiyoshi, National Center of Neurology and Psychiatry (Japan), Japan
Anna Maria Pittaluga, Università di Genova, Italy
Brian Morris, University of Glasgow, United Kingdom

***Correspondence:**
Ales Stuchlik
ales.stuchlik@fgu.cas.cz; stuchlik@biomed.cas.cz
Jan Svoboda
svobodaj@biomed.cas.cz

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *22 September 2017*
Accepted: *15 January 2018*
Published: *12 February 2018*

Citation:
Uttl L, Petrasek T, Sengul H, Svojanovska M, Lobellova V, Vales K, Radostova D, Tsenov G, Kubova H, Mikulecka A, Svoboda J and Stuchlik A (2018) Chronic MK-801 Application in Adolescence and Early Adulthood: A Spatial Working Memory Deficit in Adult Long-Evans Rats But No Changes in the Hippocampal NMDA Receptor Subunits. Front. Pharmacol. 9:42. doi: 10.3389/fphar.2018.00042

PD 60 in Long-Evans rats leads to long-term changes in working memory, but this deficit is not paralleled by changes in NMDA receptor subunits. These results support the face validity, but not construct validity of this model. We suggest that chronic treatment of adolescent and adult rats does not constitute a plausible animal model of schizophrenia.

Keywords: schizophrenia, animal model, dizocilpine, rats, chronic treatment, western blot, behavior

INTRODUCTION

Schizophrenia is a devastating neuropsychiatric disease (Owen et al., 2016), affecting all populations worldwide with a lifetime prevalence of approximately 1%. Beside, to its well-known symptoms, this disorder manifests with severe cognitive deficits. Importantly, although cognitive deficits used to be viewed as secondary symptoms, today they are considered the most stable symptom class and provide the most rigorous basis for predictions of long-term treatment outcomes (Elvevag and Goldberg, 2000; Owen et al., 2016). It has long been suspected that the glutamatergic system of the brain is involved in schizophrenia (Kantrowitz and Javitt, 2012) because many glutamatergic markers are altered in the brains of schizophrenic patients. More recently, a decreased function of glutamate receptors in schizophrenia has been combined with developmental concepts involving changes in genetic and environmental contributing to the disease to form a neurodevelopmental hypothesis of schizophrenia (Davis et al., 2016).

As no direct causes or causal treatments for schizophrenia are known, scientists often develop and evaluate animal models of schizophrenia (Jones et al., 2011) as tools for investigating mechanisms that may play a role in the actual disease and to search for novel drugs with a better risk/benefit ratio. Importantly, marked disruptions of behavioral functions similar to those found in schizophrenia can be induced in animal models using acute application of MK-801, a prototypical experimental high-affinity non-competitive antagonist of NMDA receptors. These include social deficits (low doses; Rung et al., 2005), cognitive deficits (low-to-moderate doses; van der Staay et al., 2011; Lobellova et al., 2013, Kubík et al., 2014; Svoboda et al., 2015) and toxic and experimental psychoses (higher doses, Vales et al., 2006; Lobellová et al., 2015). Chronic experiments aimed at mimicking the neurodevelopmental abnormalities have shown that these manipulations can also induce schizophrenia-like behaviors. There is a large body of evidence in rats treated with non-competitive NMDA receptor antagonists at an early postnatal age (excellently reviewed by Lim et al., 2012). However, relatively fewer studies have focused on the chronic effects of NMDA receptor antagonism in subsequent ontogenetic periods such as late adolescence or early adulthood, despite the fact that schizophrenia in human patients manifests itself most commonly in this age range (but see Li et al., 2011).

We hypothesized that repeated administration of MK-801 adolescent (starting on PD 30) and young adult rats (PD 60) would result in cognitive disturbances and changes in the levels of NMDA receptor subunits in the hippocampus, a region crucial for these functions and critically involved in schizophrenia. We sought to remedy knowledge gap on this time of treatment and test phenomenological (changes in behavior) and construct (changes in NMDA receptor subunits) axes of validity of this model.

Abbreviations: ANOVA, analysis of variance; EPM, elevated-plus maze; MK-801, dizocilpine maleate; MWM, Morris water maze; NMDA (receptors), *N*-methyl-D-aspartate (subtype of glutamate receptors); PCP, phencyclidine; PD, postnatal day; SEM, standard error of the mean; TBS, Tris-buffered saline.

MATERIALS AND METHODS

Animals

We use two common outbred rat strains, Wistar and Long-Evans. Male rats of Wistar ($n = 32$) and Long-Evans ($n = 40$) strains were obtained from the breeding colony of the Institute of Physiology of the Czech Academy of Sciences (IPHYS). They were housed in 25 cm × 30 cm × 40 cm plastic transparent cages in groups consisting of 2–4 animals in an air-conditioned animal room with constant temperature, humidity and a 12/12 h light/dark cycle. The rats were weaned at PD 28. Access to food and water was always *ad libitum*. All animal manipulations were approved by the Ministry of Agriculture committee and done according to the approved project of experiments no. 136/2013. The procedures complied with the Animal Protection Code of Czechia and the appropriate directive of the European Union (2010/63/EC).

Drugs

MK-801 (dizocilpine maleate) was obtained from Sigma–Aldrich, Czechia. The drug was dissolved in saline at a concentration of 0.5 mg/ml and injected subcutaneously in the skin fold between the shoulders at a dose of 0.5 mg/kg body weight. Control groups received subcutaneous injections of saline at a volume of 1 ml/kg. Injections were administered between 10 am and 11 am for 14 consecutive days.

Design of Study

For behavioral testing, we employed a working memory version of the MWM (Vales et al., 2006) with 15-s intervals between swims. Active place avoidance with reversal on a rotating arena (Carousel) was used to test cognitive coordination and flexibility (for review see Stuchlík et al., 2013). For assessment of anxiety-like behavior, we used an EPM. Notably, working memory, cognitive coordination and behavioral flexibility are markedly disrupted in patients with schizophrenia (Elvevag and Goldberg, 2000; Owen et al., 2016). Moreover, we analyzed levels of protein for NMDA receptor subunits in the hippocampus. We detected the GluN1, GluN2A, and GluN2B subunits by western blot analyses to assess changes in their expression.

Animals from each strain were injected with MK-801 starting at two ages: PD 30 or PD 60. Age-matched controls were given saline. All Long-Evans groups consisted of 10 animals. All Wistar groups consisted of eight animals. Animals were assigned to treatment groups randomly prior to start of experiments. Long-Evans and Wistar rats were tested in two different runs, separated by a 2-month interval; therefore, no direct comparisons of behavioral parameters between those two strains were performed.

Injections were separated by 24 h. Upon completing the injections, animals were left undisturbed for 5 days in their home cages to stabilize their behavior and for the drug to wash out, to prevent acute side effects from affecting the results. The behavioral tests were performed in the following sequence: the EPM, the working memory version of the MWM, and active place avoidance with reversal (**Figure 1**). At the age of 3 months, animals were decapitated in isoflurane anesthesia and both right and left hippocampi were dissected and prepared for western blot analysis of the levels of NMDA receptor subunits (see the section Bioanalytical Analysis below).

Behavioral Tasks

Elevated Plus Maze

The EPM is a gold standard for testing anxiety-like behavior in rodents (Haller et al., 2013). Many patients with schizophrenia report increased anxiety possibly do to the presence of positive symptoms (Temmingh and Stein, 2015), that is why we used this test as a part of behavioral battery aiming at face validity of chronic treatment with MK-801.

The EPM was a test of unconditioned avoidance behavior that involved four narrow arms elevated one meter above the floor of a dimly lit experimental room. A standard elevated plus maze apparatus was used (Pellow et al., 1985). Two opposite arms were enclosed by walls and the remaining arms were open. In the standard 5-min version of the test, rats spent more time exploring the enclosed arms than the open. Each rat was placed in the center of the maze with the head pointing toward the closed arms, and was then allowed to move freely for 5 min. Viewer (Biobserve) and Ethovision (Noldus) software was used to quantify behavior. The EPM test was administered on PD 49 in animals with the onset of treatment on PD 30, and on PD 78 in animals treated from PD 60 (**Figure 1**).

Working Memory Version of the Morris Water Maze

The working memory version of the MWM (Vales et al., 2006) permits the study of spatial working memory encoded by a single learning trial (the first swim in each session). Working memory deficit constitutes a strong and reliable phenotype for schizophrenia (Forbes et al., 2009) and reaches spatial domain (Fajnerová et al., 2014). We therefore used this task as a component of battery assessing face validity of chronic MK-801 model. The MWM was performed over 10 daily sessions consisting of four swims (trials) to a hidden platform, with a delay of 15 s between trials. The platform position was changed daily in a pseudorandom order. Each day, the platform was in a unique location; there was to interference between the sessions. Release positions were pseudo-randomized by the partial Latin square method. The MWM was administered from PD 50 in the younger age cohort and from PD 79 in the older age cohort (**Figure 1**).

Active Place Avoidance on a Rotating Arena (Carousel)

Active place avoidance with reversal in Carousel is a test of cognitive coordination and behavioral flexibility. The task has been repeatedly validated (Bures et al., 1997; Fenton et al., 1998; Czéh et al., 2001; Stuchlik and Bures, 2002; Wesierska et al., 2005; Kubík et al., 2006; Stuchlik and Vales, 2006; Petrasek et al., 2014a,b; for review see Stuchlík et al., 2013) and a typical dynamic-environment test (Stuchlik, 2014; for comparison, see Telensky et al., 2011). To our best knowledge, no other test places demands for coordinating two reference frames (arena and room frame). Cognitive coordination is significantly disrupted in schizophrenia (Phillips and Silverstein, 2003); therefore, the selection of this task was justified in this study. The testing consisted of 10 sessions (all separated by 24-h intervals). The initial five sessions were considered as acquisition sessions (with the to-be-avoided sector in arbitrary North), which were followed by five reversal sessions, with the sector position shifted by 180 degrees (arbitrary South). This shift made the task sensitive not only to cognitive coordination, but also to cognitive flexibility (Burghardt et al., 2012; Lobellova et al., 2013; Svoboda et al., 2015). Carousel maze testing was conducted during daylight hours (10 am – 4 pm).

The rotating arena (Carousel) (invented by Fenton et al., 1998 and originally described by Bures et al., 1997) was a smooth metallic arena (82 cm in diameter), enclosed with a 50-cm transparent Plexiglas wall (for details of the apparatus and procedures see Stuchlík et al., 2013). Prior to testing, conscious rats were gently implanted with a hypodermic needle, piercing the skin fold on the animal's back. The sharp end of needle was blunted and a small loop was created with tweezers, preventing the needle from slipping out and providing purchase for an alligator clip, which delivered mild electric shocks (see below). The needle implantation corresponded to subcutaneous injection in humans and did not require anesthesia. At the beginning of each session, a rat was placed on the arena, which rotated constantly at one revolution per minute. A 60-degree to-be-avoided sector was defined in the coordinate frame of the room by a computer-based tracking system (Tracker, Biosignal Group, United States), which also recorded the positions of the rat and the arena (which were both marked by infrared LED diodes) at a sampling rate of 25 Hz. Each entrance into the sector lasting more than 300 ms was punished by mild constant-current footshocks (repeated every 1200 ms until the rat left the sector) delivered by the tracking system. The intensity of the shock was individualized for each rat (0.2–0.6 mA), to ensure an escape reaction while avoiding freezing. Most rats responded appropriately to 0.2 or 0.4 mA. The footshocks were administered through a cable attached to a harness on the back of the rat and connected to the conductive subcutaneous implant. This level of shock intensity is set to be unpleasant for rats, but not painful; in the latter case, the footshock application would elicit freezing, which would have prevented the avoidance learning. Therefore, we kept the footshock on minimal possible level.

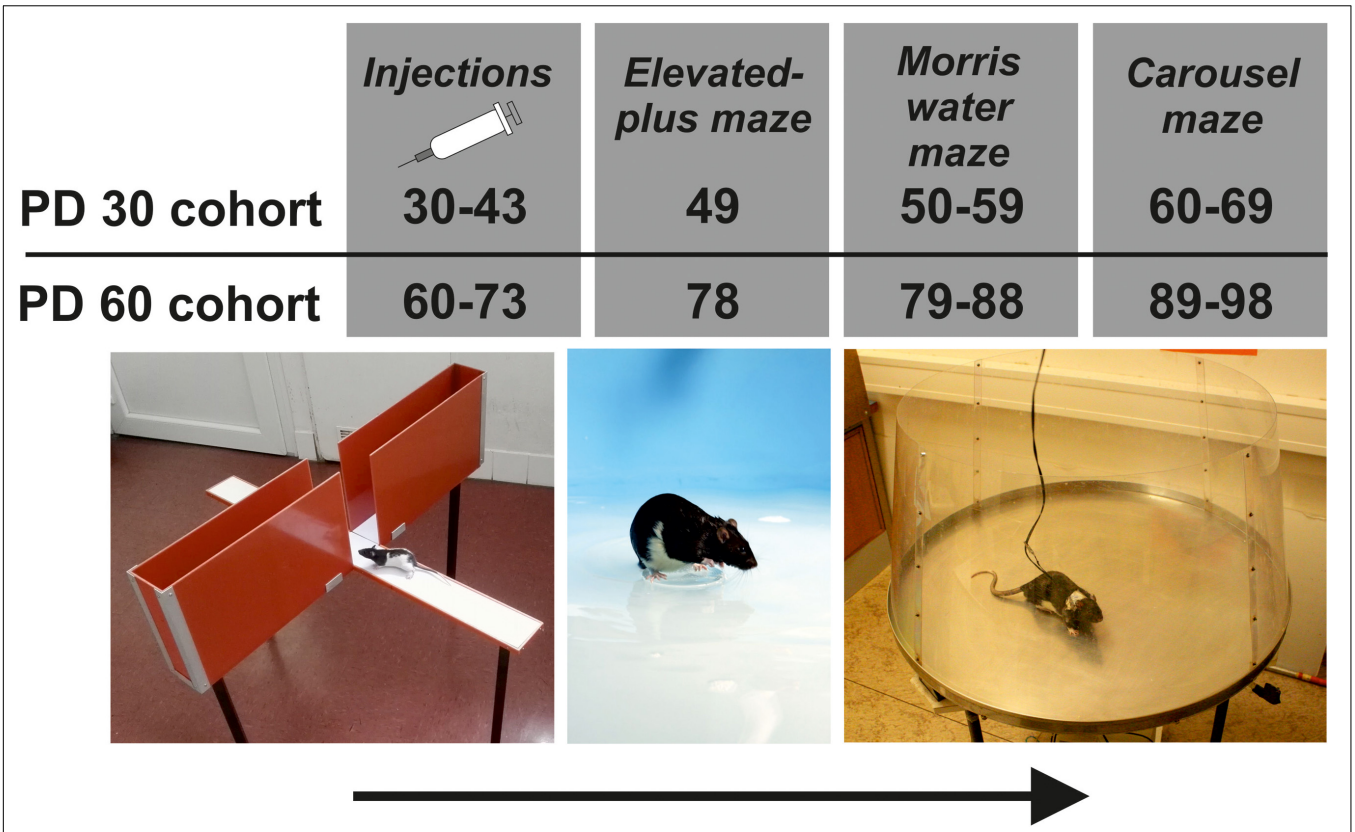

FIGURE 1 | Schematic diagram of the experimental design.

The trajectories were digitized and recorded on a PC, allowing the off-line reconstruction and analysis of the animal's trajectory and avoidance behavior with Track Analysis software (Biosignal Group, United States). Further detailed analysis and verification of the data was done in the open-source software Carousel Maze Manager (Bahník, 2014). Testing in the Carousel was conducted from PD 60 in the younger age cohort and from PND 89 in the older age cohort (**Figure 1**).

Bioanalytical Analysis

Western blot analysis was performed to determine changes in the concentration of subunits of the NMDA receptor, measuring the levels of protein expression for the GluN1, GluN2A, and GluN2B subunits. Tissue from the left and right hippocampi was collected from both the Wistar and Long-Evans rats, frozen, and stored at −80°C until analysis. Next, all samples were homogenized using a series of ultrasonic pulses (50% of max. amplitude, duration 500 ms, 8 pulses per sample; UP100H, Hielscher) with 10 mM PBS (pH 7.4) at a ratio of 1:9 and protease inhibitor cocktail (#P8340, Sigma–Aldrich). Obtained homogenates were centrifuged (#120951, SIGMA 2-16 PK) at 1000 *g* for 10 min at 4°C and the supernatant was collected. A small volume of the hippocampus lysate was used for the quantification of protein concentrations by the Lowry method (Lowry et al., 1951) with Peterson's modification (Peterson, 1977).

Before electrophoresis, samples were mixed with Laemmli loading buffer (#161-0737, Bio-Rad, Hercules, CA, United States) and heated for 20 min at 70°C. Stain-Free gradient gels (#567-8084, Bio-Rad, Hercules, CA, United States) were used for protein separation. The samples were subsequently transferred to nitrocellulose membranes (#170-4271, Bio-Rad) using a *Trans*-blot Turbo apparatus (Bio-Rad, United States). The quality of transfer and volume of protein on the membrane were determined by a ChemiDoc^{TM} Touch Imaging System (Bio-Rad, United States). Membranes were blocked in 5% non-fat milk in TBS overnight. On the subsequent day, membranes were first incubated with either the primary antibodies anti-GluN1 1:1000 (NeuroMab clone N308/48), anti-GluN2A 1:500 (NeuroMab clone N327/95) or anti-GluN2B 1:1000 (NeuroMab clone N59/36), and then washed 3x 10 min in TBS. Then, membranes were incubated for 2 h at room temperature with secondary antibody at 1: 30 000 (#115-035-174, Jackson ImmunoResearch Laboratories, Baltimore, PA, United States) for 1 h (RT) and washed with TBS as described above. A chemiluminescent substrate (Supersignal West Femto, #34096, Thermo Scientific, Waltham, MA, United States) was used for the visualization of protein with the ChemiDoc^{TM} Touch Imaging System (Bio-Rad, United States). The bands were detected and analyzed with ImageLab software. (Bio-Rad, United States). Stain-free images of total protein were used for normalization of the target proteins as described previously (Tramutola et al., 2016). When performing

normalization, detection of a housekeeping protein (for example, actin, tubulin, GAPDH) or total protein in the lines can be used. However, housekeeping proteins can vary in different tissues, at different ages, but also because of experimental manipulations (Fukazawa et al., 2003; Li and Carmichael, 2006). For these reasons, we chose normalization to total protein. The above-mentioned stain-free method was used to determine the total protein in the lanes. The principle of the method is based on modification of the tryptophan amino acid by the compound trihalo, resulting in a very marked increase in fluorescence after activation by UV light. This method is comparable to protein staining with SYPRO Ruby, Coomassie Blue or silver stain (Gilda and Gomes, 2013). For illustration, see the stain-free images in Supplementary Material.

Data Analysis and Statistics

Analysis of the Behavioral Parameters

In the elevated plus-maze, the following parameters were measured: time spent in the open arms, closed arms, and central platform, head dipping and risk assessments (the latter two are not reported, as no effects were seen). In the MWM, latency and total distance to reach the platform were recorded. In the Carousel, we measured the number of entrances into the to-be-avoided sector (number of errors) and total distance walked during a session (computed as the cumulative total distance of data points measured at 1-s intervals; this sampling eliminates non-locomotor movements such as shivering). We also recorded the maximum time between two entrances (maximum time avoided) and the latency to the first entrance to the to-be-avoided sector in a given session (latency to the first error). However, since these parameters generally correlate highly with the number of errors, their analysis did not provide any additional information value; these data are not included here. In all these behavioral experiments, statistical analyses were performed separately for each strain as both strains were testing in separate batches. Data from the elevated plus-maze were assessed using two-way ANOVA with age and MK-801 treatment as main factors. To avoid three-way ANOVAs in water maze and active place avoidance in which repeated measures add another factor of complexity, we selected a parameter that well characterizes the extent of learning. In water maze, we summed the latency and total distance from swims 2 to 4 and averaged them across the 10 days of training. In active place avoidance, we summed number of entrances and total distance across the 5 days of acquisition. We also evaluated these two parameters in 1st day of reversal when they best reflect behavioral flexibility. Two-way ANOVA was conducted with treatment (MK-801, saline) and age (PD30, PD60) as the main factors. When appropriate, ANOVA was followed by Sidak's *post hoc* test. All calculations were performed using GraphPad Prism 7.0, with a level of acceptance set at $P < 0.05$. Data are reported in the figures as means ± SEM.

Analysis of Biochemical Parameters

Images of target proteins and stain-free images of total protein were created on the ChemiDoc^{TM} Touch system and analyzed in ImageLab. The values were taken from the left and right hippocampi, but since there were no differences between them, they were pooled together for subsequent analyses. The measured values were tested for outliers by calculating a z score, and consequently the values having a z score greater than 2 were excluded. We used 14–16 samples in each group. For statistical analysis we used Statistica 9 (StatSoft) and graphs were created using GraphPad Prism 5.0. Differences between groups were evaluated by a two-way ANOVA with treatment and age as independent factors; statistically significant differences are reported at $P < 0.05$. Data are reported as means ± SEM total protein normalized intensity.

RESULTS

Repeated Administration of MK-801 Causes Severe Qualitative Acute and Long-term Effects

In both strains, both age groups of rats administered with 0.5 mg/kg MK-801 displayed acute behavioral changes as expected. Since 0.5 mg/kg is a relatively high dose, we observed the onset of restlessness 5 min after application followed by hyperactivity (running near the wall, sniffing in the corners, salivation) and motor disturbances (inability to stand upright, falling and crawling). Some rats were hypoactive and even had signs of ataxia. This type of behavior typically diminished 1–2 h after MK-801 administration. One week after injection, three Long-Evans rats exhibited increased aggressiveness, and therefore were placed in cages separately and were not included in behavioral and bioanalytical testing.

Behavioral Results

Repeated MK-801 Led to Mild Long-term Anxiety-Like Behavior in Adolescent Long-Evans Rats

Wistar rats

Data obtained in the EPM suggests that MK-801 exerted no effects on anxiety-like behavior in Wistar rats. As **Figure 2** illustrates, there was no difference between saline and MK-801 injected groups in neither age when evaluating the time spent in the center [$F(1,28) = 2.988$], open arms [$F(1,28) = 0.01$] or closed arms [$F(1,28) = 1.577$]. However, on average PD30 rats spent less time in the center than PD60 rats [effect of age: $F(1,28) = 6.125$, $P = 0.0196$].

Long-Evans rats

When analyzing behavior of Long-Evans rats, we found significant effect of MK-801 in the time spent in closed arms [$F(1,36) = 5.737$, $P = 0.0219$] and significant age vs. treatment interaction in time spent in the center [$F(1,36) = 8.259$, $P = 0.0068$]. *Post hoc* tests revealed it was due to different effects in PD30 groups. These results indicate elevated anxiety-like behavior in Long-Evans rats in the younger age cohort. On the other hand, time spent in open arms was not affected by MK-801 treatment in Long-Evans rats [$F(1,36) = 1.556$, $P = 0.2204$].

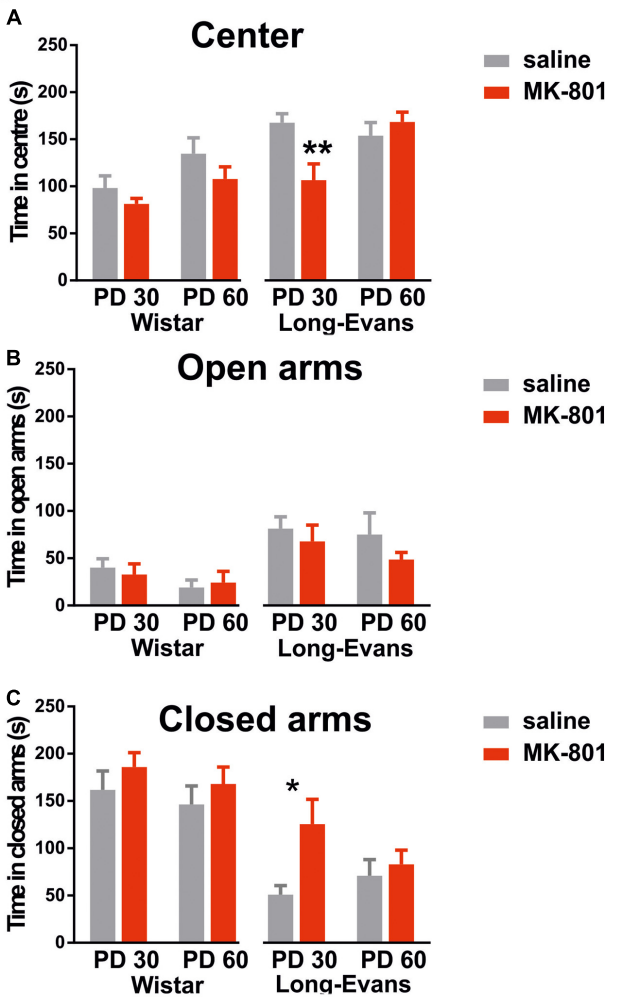

FIGURE 2 | Performance in the elevated plus maze evaluated by total time spent in the central part **(A)**, open arms **(B)**, and closed arms **(C)**. Comparing MK-801 and saline-treated rats in each cohort yielded significant differences only for PD 30 Long-Evans rats in entering the center, and for PD 30 Long-Evans rats in entering the closed arms. Group means ± SEM (*$P < 0.05$, **$P < 0.01$).

Repeated MK-801 Induced Long-term Impairments of Working Memory in Adult Long-Evans Rats

Wistar rats

To quantify performance in the working memory version of the MWM, we summed the latency and total distance from swims 2 to 4 and averaged them across the 10 days of training. As can be seen in **Figure 3**, MK-801 elicited no effects in Wistar rats. Two-way ANOVA failed to find an effect of MK-801 [latency: $F(1,28) = 0.8502$, $P = 0.36$; distance: $F(1,28) = 0.8165$, $P = 0.37$] and age [latency: $F(1,28) = 0.008$, $P = 0.93$; distance: $F(1,28) = 0.056$, $P = 0.82$].

Long-Evans rats

In contrast, pattern in Long-Evans rats was more complex. Analyzing latency, two-way ANOVA failed to see an effect of MK [$F(1,30) = 0.96$, $P = 0.33$] but showed main effect of age [$F(1,30) = 10.52$, $P = 0.003$] and interactions [$F(1,30) = 7.99$, $P = 0.008$]. Then Sidak's *post hoc* test confirmed significantly increased latency in MK-801-treated PD 60 rats compared to PD 60 controls. Similar results were obtained when analyzing path: Main effect of MK-801 [$F(1,33) = 4.13$, $P = 0.05$], age [$F(1,33) = 0.39$, $P = 0.54$], and interaction [$F(1,33) = 7.71$, $P = 0.009$]. Subsequent *post hoc* test confirmed elevated path in PD 60 MK-801 rats compared to PD60 controls.

Repeated MK-801 Failed to Induce Deficits in Acquisition and Reversal Learning in the Carousel

Number of errors (entrances) reflects the ability of properly locating the to-be-avoided place on the arena. We summed number of entrances across the five sessions of acquisition to get an overall parameter of acquisition. Then we analyzed data from first reversal session as this time point allows for evaluating the ability of cognitive flexibility.

Wistar rats

Despite **Figure 4** indicates that PD 60 Wistar rats accumulated lower number of entrances during acquisition, two-way ANOVA failed to see an effect of age [$F(1,28) = 3.534$, $P = 0.07$]. Furthermore, there was neither effect of MK-801 administration [$F(1,28) = 0.3092$, $P = 0.58$] nor significant treatment vs. age interaction – [$F(1,28) = 0.175$, $P = 0.6789$]. Similarly, cognitive flexibility as measured by number of entrances in the 1st day of reversal was not affected by MK-801 administration in Wistar rats [$F(1,33) = 0.013$, $P = 0.91$] or age [$F(1,28) = 3.534$, $P = 0.0706$]; interaction [$F(1,28) = 0.175$, $P = 0.6789$].

Long-Evans rats

As **Figure 4** illustrates, Long-Evans rats performed equally during acquisition at both ages [$F(1,33) = 2.723$, $P = 0.11$] despite MK-801 administration [$F(1,33) = 0.01319$, $P = 0.9093$] measured by a summed number of entrances during acquisition. Surprisingly, a two-way ANOVA found a significant effect of age [$F(1,33) = 8.782$, $P = 0.0056$] and effect of age vs. MK-801 interaction [$F(1,33) = 3.973$, $P = 0.05$] in the 1st day of reversal resulting from decreased number of entrances in Long-Evans rats treated with MK-801 from PD 60 (Sidak's *post hoc* test, $P = 0.03$). Besides the number of entrances, we also evaluated the total distance moved during a session to investigate the effects of repeated administration of MK-801 on overall locomotion, but two-way ANOVA did not reveal a significant effect of MK-801 or age in any experimental condition in either strain or any cohort.

Repeated MK-801 Failed to Affect the Levels of NMDA Receptors Subunits in the Hippocampus

Table 1 shows the mean of values of protein concentrations (μg/μl). Hippocampal expression of NMDA subunits (GluN1, GlunN2A, and GluN2B) was analyzed for both strains independently with using a two-way ANOVA (see **Figures 5**, **6**).

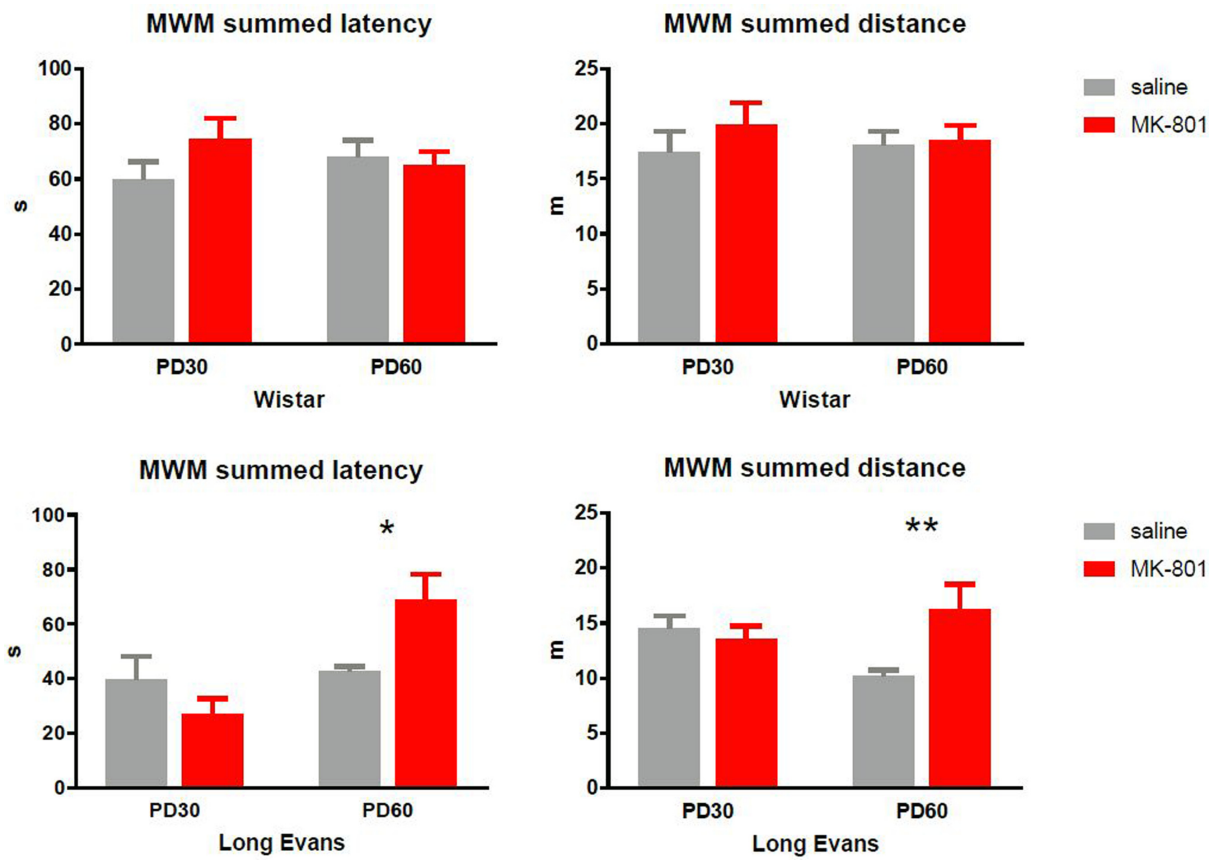

FIGURE 3 | Latency and path to the platform (distance) in the water maze summed for swim 2, 3, and 4, and averaged across the 10 days of training. In the Long-Evans PD 60 cohort, MK-801 treated rats did not search for the platform on repeated swims as efficiently as saline treated rats (*$P < 0.05$, **$P < 0.01$). Group means ± SEM.

Wistar Rats

Analysis of expression of NMDA receptor subunits in Wistar rats showed no significant changes. Expression of GluN1 showed no significant main effects of treatment group [$F(1,60) = 0.113$, $P = 0.738$] and age [$F(1,60) = 2.556$, $P = 0.11723$] neither age × treatment of interaction [$F(1,60) = 0.141$, $P = 0.709$]. Expression of GluN2A showed no significant changes main effects of age [$F(1,60) = 2.5006$, $P = 0.11906$] and treatment [$F(1,60) = 0.8581$, $P = 0.358$] neither age × treatment interaction [$F(1,60) = 0.2149$, $P = 0.6447$]. Expression of GluN2B showed no significant main effects, treatment [$F(1,60) = 0.185$, $P = 0.668$] and age [$F(1,60) = 0.083$, $P = 0.775$], neither interaction treatment × age [$F(1,60) = 2.889$, $P = 0.094$].

Long-Evans Rats

Expression of GluN1 subunits in Long-Evans rats showed no significant main effect of treatment [$F(1,58) = 0, 41$, $P = 0.524$], but a significant main effect of age for administration [$F(1,58) = 10.062$, $P = 0.002$] and significant effect of treatment × age interaction [$F(1,58) = 6.4068$, $P = 0.014$]. Tukey *post hoc* test showed a significant decrease of GluN1 between PD 30 and PD 60 groups with MK-801 ($P = 0.0013$) and between control group PD 30 and MK-801 PD60 ($P = 0.049$). Expression of GluN2B showed no significant main effects of age [$F(1,52) = 2.6417$, $P = 0.11014$] and treatment [$F(1,52) = 0.375$, $P = 0.543$], but a significant treatment × age interaction [$F(1,52) = 6.1454$, $P = 0.01646$]. Tukey *post hoc* test showed a decrease of GluN2B between PD 30 and PD 60 after MK-801 administration ($P = 0.045$). Expression of GluN2A showed no significant main effect of treatment [$F(1,52) = 0.298$, $P = 0.588$], age [$F(1,52) = 0.014$, $P = 0.907$], or interaction [$F(1,52) = 0.80640$, $P = 0.37333$]. However, not significant differences between MK-801- and saline-treated PD 30 and PD 60, respectively, Long-Evans rats were found.

DISCUSSION

General Remarks

In this study, we evaluated the effects of a 2-week repeated treatment with the NMDA antagonist MK-801 given in the adolescent and early adulthood periods in Wistar and Long-Evans rats. We detected an elevation of anxiety-like behaviors, measured by time spent in the center and closed arms but not open arms of the elevated plus maze, in Long-Evans rats treated with MK-801 from an adolescent age. Moreover,

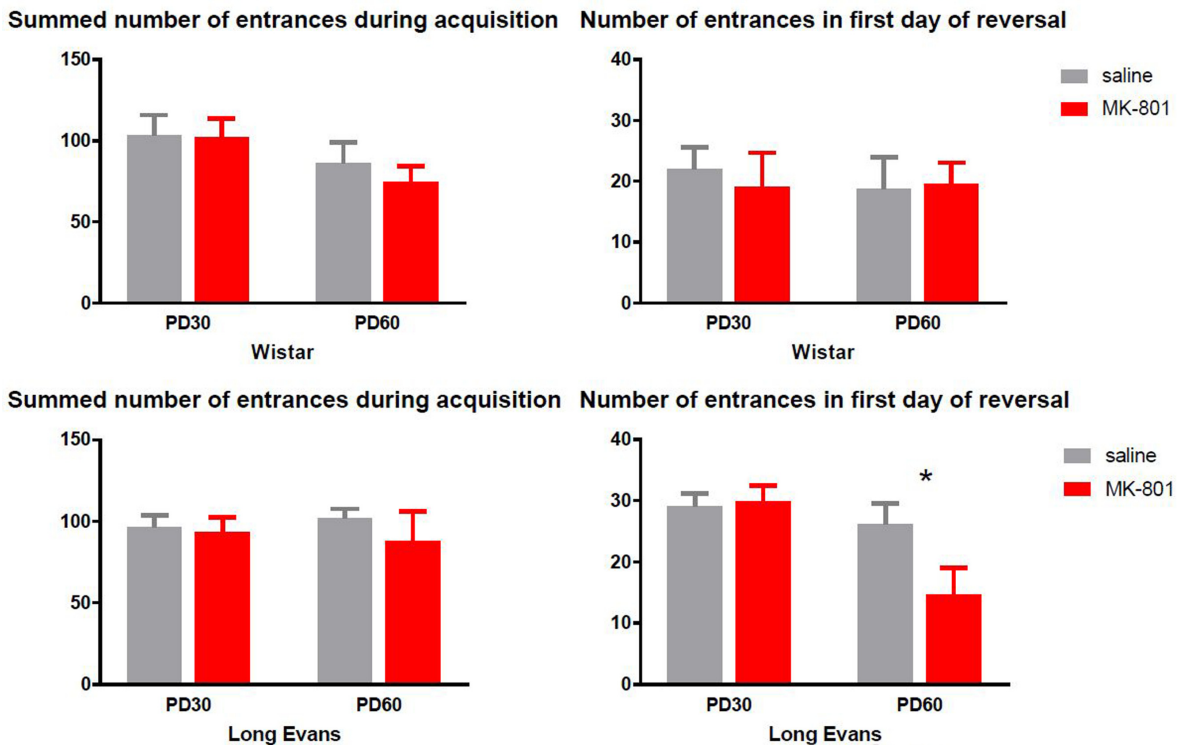

FIGURE 4 | Performance in active place avoidance evaluated as the summed number of entrances into the to-be-avoided sector during 4 days of acquisition (left graphs) and 1st day of reversal (right graphs). Differences between MK-801 and saline treated rats were statistically significant only during reversal in the Long-Evans PD 60 cohort, with MK-801 administered rats to be superior to saline controls (*$P < 0.05$). Group means ± SEM.

we found a significant impairment of working memory tested in the MWM in Long-Evans rats treated at the early adult age (from PD 60). No differences were found in cognitive learning and flexibility tested in the Carousel. There were also no significant changes in the expression of NMDA receptor subunits because of MK-801 in any strain and age. Acute administration of NMDA receptor antagonists is used by recreational drug users and in animal models and it can mimic some aspects of psychosis. Abuse of NMDA antagonists in adolescence is associated with higher overall effect, risks for developing psychosis and dependence compared to adult use in human subjects. This notion was demonstrated in rats too (Rocha et al., 2017) where adolescent rats displayed more pronounced behavioral activation to single dose of PCP and ketamine. Contrarily, repeated intermittent dosing of these drugs showed more pronounced sensitization effect in adults than in adolescent rats.

TABLE 1 | Mean of total protein concentration in samples for Western blot analyses.

Groups		Concentration of proteins in samples (μg/μl)		Count of samples
		Mean	SEM	
Wistar	Control PD 30	7.56	0.31	$n = 16$
	Control PD 60	8.02	0.13	$n = 16$
	MK-801 PD 30	7.57	0.35	$n = 16$
	MK-801 PD 60	9.18	0.25	$n = 16$
Long-Evans	Control PD 30	7.48	0.30	$n = 20$
	Control PD 60	6.23	0.34	$n = 20$
	MK-801 PD 30	6.90	0.36	$n = 18$
	MK-801 PD 60	5.42	0.49	$n = 10$

Data was obtained by using a modificated Lowry's method.

Chronic administration of MK-801, PCP and ketamine in rodent models are currently used to achieve long-term changes in behavior and neurotransmission. Abdul-Monim et al. (2006) reported that female rats trained to solve a simple task failed to complete the task again after chronic PCP administration. Further, the administration of atypical antipsychotics (ziprasidone, olanzapine, clozapine) improved performance, but classical antipsychotics did not have the same beneficial effect (Abdul-Monim et al., 2006). In another study, rats performed significantly worse in the MWM 2 weeks after subchronic MK-801 administration, and at the same time did not differ in weight or locomotor activity (Li et al., 2011).

Behavioral Changes after Repeated MK-801 Administration

No clear effects on anxiety, working memory, or spatial learning were detected in Wistar PD 30 or PD 60 rats given MK-801

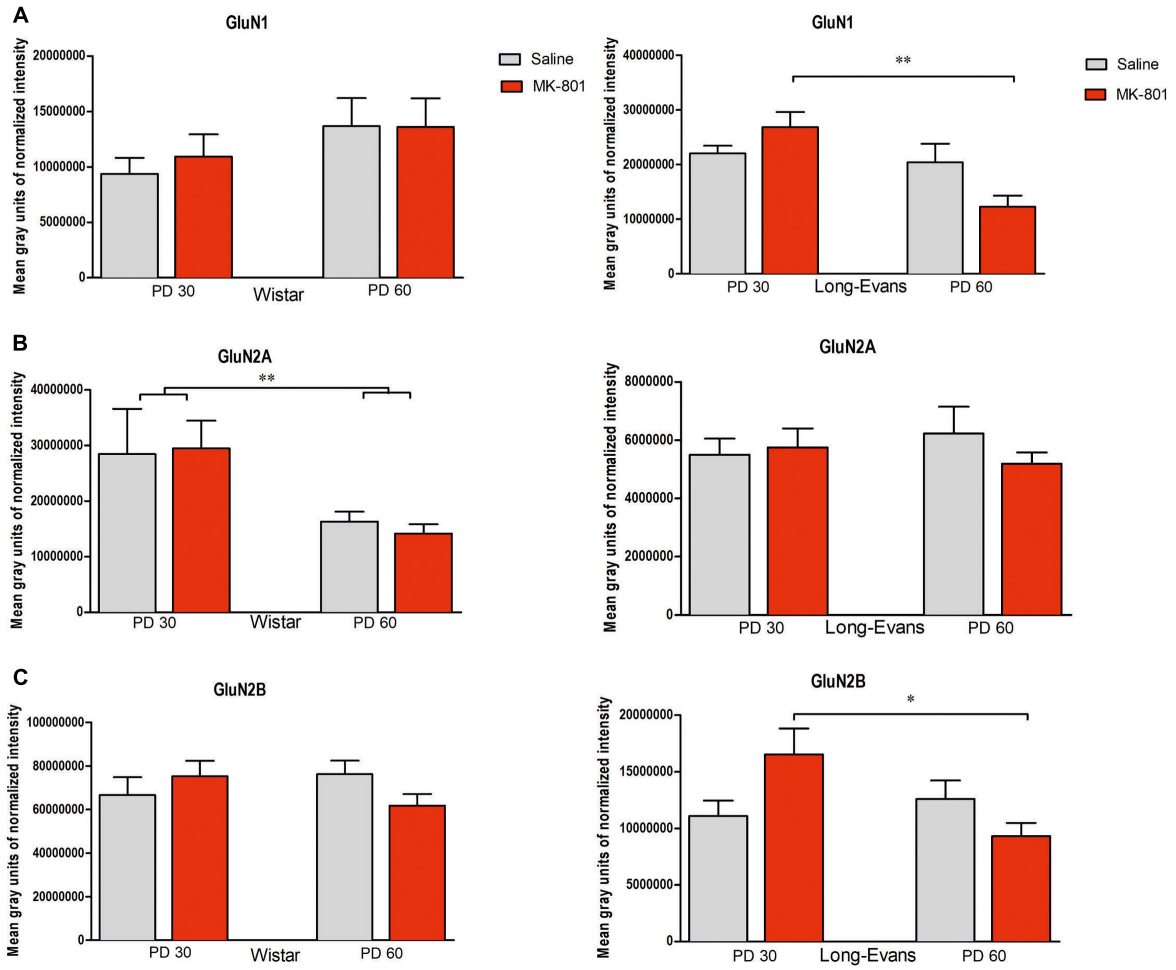

FIGURE 5 | Expression of NMDA subunits in the hippocampus: **(A)** GluN1 subunit, **(B)** GluN2A subunit **(C)** GluN2B subunit: The charts show the two intervals of the start of administration (PD 30 and PD 60). Strains of rats are marked as Long-Evans (LE) and Wistar (W). The data show the mean level of chemiluminescence after total protein normalization (group means ± SEM). $^{\#}P < 0.05$ compared to controls of the same strain and age. $^{*}P < 0.05$, $^{**}P < 0.01$; comparison between age cohorts.

for 2 weeks. In contrast, Long-Evans rats were more affected by this treatment. Our results show that Long-Evans rats from the older age group, i.e., PD 60 at the onset of treatment display cognitive impairments, whereas adolescent Long-Evans rats are susceptible to elevations in anxiety-like behaviors, induced by chronic NMDA receptor antagonism.

Anxiety-Like Behavior

We did not detect any alterations in anxiety-like behavior in Wistar rats. Long-Evans rats administered MK-801 on PD 30 displayed higher anxiety levels in the EPM by spending more time in the closed arms than the control group. However, there was no significant change in the time spent in open arms. Studies on long-term changes in anxiety-like behavior due to MK-801 and other glutamatergic psychotomimetics are rather scarce. In contrast to the increased anxiety in PD 30 Long-Evans rats found in this study, Kocahan et al. (2013) and Latysheva and Rayevsky (2003) found decreased levels of anxiety-like behavior in the EPM in Wistar rats treated from PD 7 to PD 10 with 0.25 mg/kg and from PD 7 to PD 49 with 0.05 mg/kg. However, Baier et al. (2009) reported increased anxiety-like behavior on PD 90 in the EPM in Wistar rats after 0.25 mg/kg of MK-801 administered from PD 6 to PD 21. These discrepancies are likely caused by a different dose and timing of applications, but our results imply a susceptibility to elevated anxiety in Long-Evans rats treated in adolescence.

Spatial Working Memory in the MWM and Cognitive Learning in the Carousel

Interestingly, testing in the MWM revealed an impairment of working memory only in older PD 60 MK-801-treated Long-Evans rats compared to saline-treated subjects. Rats given MK-801 were still able to learn the task but at a slower rate. Active place avoidance testing did not reveal any difference between MK-801-treated and saline-treated rats 30 days after application, with no effects found on cognitive coordination

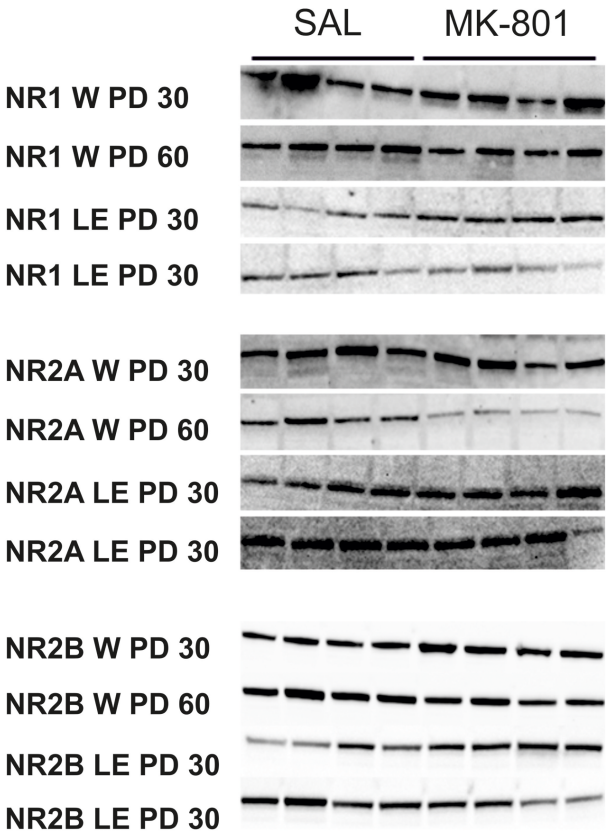

FIGURE 6 | Examples of Western blot of the hippocampal supernatant showing expression of the NMDA subunits (GluN1 ∼100 kDa, GluN2A∼170 kDa, GluN2B ∼180 kDa) at two age intervals (PD 30, PD 60) in Long-Evans and Wistar rats. Control group samples are in columns 1–4 and MK-801 samples are in the columns 5–8.

and flexibility. It should be noted that overall performance of both control and MK-801 rats (Vales and Stuchlik, 2005; Stuchlik et al., 2008), which implies that chronic stress caused by injections in all groups may have negatively affected their performance.

Previously published studies have reported contrasting effects of chronic MK-801 treatment in the postnatal and early adolescent periods on cognition in the spatial and non-spatial domains. McLamb et al. (1990) observed no impairment in a water maze after 0.2 mg/kg MK-801 treatment administered from PD 9 to PD 15 in Fisher-344 rats. Kocahan et al. (2013) failed to find any deficit in an inhibitory avoidance task in Wistar rats treated in the early postnatal age and tested at adolescence (application: PD 7 – PD 10; testing at PD 35 – PD 45). Rats treated two times a day for 7 days with 0.5 mg/kg MK-801 in adulthood were not affected by the treatment in a variable-delayed alternation task or in a T-maze 36 h after administration (Seillier and Guiffrida, 2009). However, other studies have shown clear impairments in spatial memory after chronic MK-801 application, further supporting our data. After 0.25 mg/kg MK-801 given from PD 8 to PD 19, Wistar rats displayed a slower learning rate in a water maze task in adulthood (Gorter and de Bruin, 1992). Latysheva and Rayevsky (2003) treated Wistar rats with 0.05 mg/kg MK-801 from PD 7 to PD 49, and found impaired performance in a complex maze 5 days after treatment. Kawabe and Miyamoto (2008) found impairment in a delayed non-matching-to-position task in adulthood after 0.2 mg/kg and 0.4 mg/kg MK-801 applications two times a day from PD 7 to PD 20 in Wistar rats. In mice, 0.1 mg/kg MK-801 treatment administered from PD 3 to PD 17 worsened performance in the MWM (Elhardt et al., 2010). Finally Li et al. (2011) observed a disruption of spatial working memory in the MWM after 2-week administration OF MK-801 to adolescent Sprague-Dawley rats (starting on PD 28), further indirectly supporting our data although with a different age and strain. Again, different doses and time of administration precludes direct comparison or data of ours and other authors, but the results show a spatial working memory deficit due to MK-801 treatment in young adult Long-Evans rats, supporting the face validity of this treatment in this strain.

Locomotor Activity

We did not observe hyperlocomotion in the MWM or active place avoidance after repeated MK-801 treatment, though such an effect has been seen after the acute administration of the same dose (Hargreaves and Cain, 1995; Lobellova et al., 2013; Svoboda et al., 2015). Hyperlocomotion is thought to correlate with the positive symptoms of schizophrenia and is one of the well-characterized effects of chronic MK-801 treatment (Lim et al., 2012). Kocahan et al. (2013) observed increased spontaneous locomotor activity in an open-field test in Wistar rats after 0.25 mg/kg MK-801 given from PD 7 to PD 10. Facchinetti et al. (1993) also observed hyperlocomotion in an open-field test in Wistar rats after the administration of increasing 0.5 – 1 mg/kg doses from PD 1 to PD 22, and this increased activity lasted until PD 60. After 0.25 mg/kg MK-801 administered from PD 6 to PD 21, Schiffelholz et al. (2004) detected increased spontaneous activity on PD 30, but from PD 60 rats displayed hypolocomotion lasting until PD 180. In contrast, however, numerous other authors have reported no effects of early-life MK-801 treatment on spontaneous locomotor activity in rats (Stefani and Moghaddam, 2005; Kawabe et al., 2007; Uehara et al., 2009). McLamb et al. (1990) reported no effects of 0.2 mg/kg MK-801 administered from PD 9 to PD 15 in Fischer-344 rats on locomotor activity in the MWM. The absence of locomotor changes in our model suggests that it does not constitute a model of positive symptoms of schizophrenia.

Effects of Repeated Administration of MK-801 on NMDA Receptor Subunits

We did not detect any significant changes in the expression of GluN1, GluN2A, or GluN2B subunits in MK-801-treated Wistar or Long-Evans rats compared with control groups. We only observed differences between different times of administration of MK-801. In accordance with our data, Wang et al. (1999) failed to detect any change in GluN1 subunit expression in the hippocampus of rats chronically administered with PCP, and

only found an increase in the GluN1 subunit in the forebrain. Chronic PCP given to adult mice resulted in an increased number of binding sites for MK-801 shortly after treatment (Newell et al., 2007). However, 14 days after treatment the number of MK-801 binding sites decreased significantly, especially in the hippocampus. These results are in partial accordance with our present data. However, Oh et al. (2001) reported increased numbers of the GluN1 subunit in CA1 of the hippocampus after intracerebroventricular infusion with MK-801 in Sprague-Dawley rats. Anastasio and Johnson (2008) reported an increased number of GluN1 and GluN2B subunits induced by translocation from the endoplasmic reticulum to the membrane after acute PCP administration. In contrast, repeated administration of PCP led to the increased *de novo* synthesis of NMDA subunits. It has to be pointed out that the amounts of protein for NMDA receptor subunits might not be equal to the number of active receptors, which may contributed to the negative findings reported in this study. The changes between Long-Evans rats treated from PD 30 vs. PD 60 may have been a result of different brain responses in relation to the age of the rats during the administration of MK-801. Matta et al. (2013) described the disruption of developmental changes in NMDA receptors induced by giving MK-801 during ontogenesis. Age-dependent differences may also occur due to different NMDA currents in immature neurons compared to fully mature neurons and due to the different ontogenetic development of individual structures (Wang and Gao, 2009; Rotaru et al., 2011; Nakazawa et al., 2017). GluN2B and GluN2A subunits of the NMDA receptor show varied expression during ontogenetic development. The GluN2B subunit is most highly expressed in the first PDs and its concentration gradually decreases, while GluN2A concentrations are low after birth and gradually rise until PD 21, when they reach levels comparable with those in adulthood (Wenzel et al., 1997). Our finding that in MK-801-treated animals the levels of GluN1 and GluN2B are changed due to age of treatment most likely represent the developmental changes in sensitivity of NMDA receptor system to this drug. The absence of MK-801-induced effects also decreases the construct axis of validity of this model.

Limitations of the Study

This study has a few limitations. Notably, both strains were testing in different batches as mentioned in the section "Materials and Methods," preventing their direct comparison. In addition, both age cohorts underwent their behavioral testing at different periods of their development (PD 49 – PD 69 vs. PD 78 – PD 97), but intended to keep the interval between administration of the drugs and behavioral testing constant to allow for comparison. Repeated drug administration in the adolescent period raises concerns regarding the correct physical development of the animal. However, in this study, rats treated with MK-801 did not differ in total health status or reactions to environmental stimuli during behavioral testing (visual observations; data not shown) except the three rats that exhibited aggressiveness that were excluded from the study.

CONCLUSION

Chronic treatment with the NMDA antagonist MK-801 impaired working memory only in Long-Evans rats treated in early adulthood with application starting from PD 60. Suggestively elevated anxiety was found in Long-Evans rats treated at adolescent age. This data support the face validity of this treatment as potential animal model of cognitive deficits due to chronic experimental psychosis. However, no significant effects were observed in expression of NMDA subunits by Long-Evans or Wistar strains at any age. These finding did not support the construct validity of this model. In conclusion, our results suggest that despite a working memory deficit and elevated anxiety in one of the strains, this dose, timing and period of treatment with MK-801 does not constitute a plausible model of schizophrenia-like phenotypes.

AUTHOR CONTRIBUTIONS

All authors contributed to writing of the manuscript. HS, MS, and VL conducted the behavioral study. LU and GT conducted the Western blot. AS conceived the study and provided the scientific leadership.

ACKNOWLEDGMENTS

This work was supported mainly by AZV 17-30833A. Student's support was provided by GAUK 248915. Institutional support for IPHYS was provided by RVO: 67985823 and by Academic CZ-PL bilateral mobility project PAN-17-07. Institutional support for National Institute of Mental Health was provided by the grant LO1611 from the MEYS CR (NPU I). Partial support came from structural funds of European Union: OPPK Microscopic System CZ.2.16/3.1.00/28034, OPPK BrainView CZ.2.16/3.1.00/21544 and MEYS CR (LM2015062) Czech-BioImaging. The experiments used in this study were approved by the Committee for proper procedures in animals and welfare of the Institute of Physiology, Czech Academy of Sciences and by the Resort Committee of the Czech Academy of Sciences (Project of Experiments No. 136/2013). We thank Hana Brozka for the comments on early draft versions, Michaela Fialova, Jindrich Kalvoda, and Antonina Zahalka for their technical support, Vladimira Markova and Barbara Stuchlikova for the editing work and David W. Hardekopf for proofreading. All rights reserved. We would also like to thank Jana Novakova (Bio-Rad, Czechia) for providing the ChemiDoc™ Touch Imaging System for Western blot analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.00042/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CORRECTION
published: 20 June 2018
doi: 10.3389/fphar.2018.00693

Corrigendum: Chronic MK-801 Application in Adolescence and Early Adulthood: A Spatial Working Memory Deficit in Adult Long-Evans Rats But No Changes in the Hippocampal NMDA Receptor Subunits

Libor Uttl[1,2], Tomas Petrasek[3], Hilal Sengul[3,4], Marketa Svojanovska[3], Veronika Lobellova[3], Karel Vales[2,3], Dominika Radostova[3,5], Grygoriy Tsenov[1], Hana Kubova[1], Anna Mikulecka[1], Jan Svoboda[3] and Ales Stuchlik[3]**

[1] Department of Developmental Epileptology, Institute of Physiology, Czech Academy of Sciences, Prague, Czechia, [2] Department of Experimental Neurobiology, National Institute of Mental Health, Klecany, Czechia, [3] Department of Neurophysiology of Memory, Institute of Physiology, Czech Academy of Sciences, Prague, Czechia, [4] Radboud Institute for Molecular Life Sciences, Radboud University, Nijmegen, Netherlands, [5] Second Faculty of Medicine, Charles University, Prague, Czechia

Keywords: schizophrenia, animal model, dizocilpine, rats, chronic treatment, western blot, behavior

OPEN ACCESS

Edited and reviewed by:
Frontiers in Pharmacology Editorial Office, Frontiers Media SA, Switzerland

Correspondence:
Jan Svoboda
svobodaj@biomed.cas.cz
Ales Stuchlik
ales.stuchlik@fgu.cas.cz;
stuchlik@biomed.cas.cz

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *04 June 2018*
Accepted: *07 June 2018*
Published: *20 June 2018*

Citation:
Uttl L, Petrasek T, Sengul H, Svojanovska M, Lobellova V, Vales K, Radostova D, Tsenov G, Kubova H, Mikulecka A, Svoboda J and Stuchlik A (2018) Corrigendum: Chronic MK-801 Application in Adolescence and Early Adulthood: A Spatial Working Memory Deficit in Adult Long-Evans Rats But No Changes in the Hippocampal NMDA Receptor Subunits. Front. Pharmacol. 9:693. doi: 10.3389/fphar.2018.00693

A corrigendum on

Chronic MK-801 Application in Adolescence and Early Adulthood: A Spatial Working Memory Deficit in Adult Long-Evans Rats But No Changes in the Hippocampal NMDA Receptor Subunits

by Uttl, L., Petrasek, T., Sengul, H., Svojanovska, M., Lobellova, V., Vales, K., et al. (2018). Front. Pharmacol. 9:42. doi: 10.3389/fphar.2018.00042

There is an error in the Funding statement. The correct number for **OPPK CZ.2.16/3.1.00/** is **OPPK Microscopic System CZ.2.16/3.1.00/28034**. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

The original article has been updated.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 10 September 2018
doi: 10.3389/fphar.2018.01023

Delay-Dependent Impairments in Memory and Motor Functions After Acute Methadone Overdose in Rats

Leila Ahmad-Molaei[1], Hossein Hassanian-Moghaddam[2,3], Fariba Farnaghi[4], Carlos Tomaz[5] and Abbas Haghparast[1]**

[1] Neuroscience Research Center, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [2] Department of Clinical Toxicology, Loghman-Hakim Hospital, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [3] Social Determinants of Health Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [4] Department of Pediatric Clinical Toxicology, Loghman-Hakim Hospital, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran, [5] Neuroscience Research Program, CEUMA University, São Luís, Brazil

Methadone is used as a substitution drug for the treatment of opioid dependence and chronic pain. Despite its widespread use and availability, there is a serious concern with respect to the relative safety of methadone. The purpose of this study was to characterize how acute methadone overdose affects the cognitive and motor performance of naïve healthy rats. The methadone overdose was induced by administering an acute toxic dose of methadone (15 mg/kg; ip; the equivalent dose of 80% of LD_{50}) to adolescent rats. Resuscitation using a ventilator pump along with a single dose of naloxone (2 mg/kg; ip) was administered following the occurrence of apnea. The animals which were successfully resuscitated divided randomly into three apnea groups that evaluated either on day 1, 5, or 10 post-resuscitation (M/N-Day 1, M/N-Day 5, and M/N-Day 10 groups) in the Y-maze and novel object memory recognition tasks as well as pole and rotarod tests. The data revealed that a single toxic dose of methadone had an adverse effect on spontaneous behavior. In addition, Recognition memory impairment was observed in the M/N-Day 1, 5, and 10 groups after methadone-induced apnea. Further, descending time in the M/N-Day 5 group increased significantly in comparison with its respective Saline control group. The overall results indicate that acute methadone-overdose-induced apnea produced delay-dependent cognitive and motor impairment. We suggest that methadone poisoning should be considered as a possible cause of delayed neurological disorders, which might be transient, in some types of memory or motor performance in naïve healthy rats.

Keywords: methadone, naloxone, learning and memory performance, motor coordination, overdose, rat

OPEN ACCESS

Edited by:
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Reviewed by:
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***Correspondence:**
Carlos Tomaz
ctomaz@ceuma.br; ctomaz@unb.br
Abbas Haghparast
haghparast@sbmu.ac.ir; haghparast@yahoo.com

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *27 January 2018*
Accepted: *23 August 2018*
Published: *10 September 2018*

Citation:
Ahmad-Molaei L, Hassanian-Moghaddam H, Farnaghi F, Tomaz C and Haghparast A (2018) Delay-Dependent Impairments in Memory and Motor Functions After Acute Methadone Overdose in Rats. Front. Pharmacol. 9:1023. doi: 10.3389/fphar.2018.01023

INTRODUCTION

Methadone is a long-acting, synthetic mu-opioid agonist having multiple actions and pharmacologic properties that are similar to morphine (Barbosa Neto et al., 2015). Methadone has long been used for the treatment of opioid dependence and detoxification or maintenance in cases of opioid addiction because of its long efficacy and low cost (Kleber, 2007). In addition, like other opioids such as buprenorphine, fentanyl, morphine, and oxycodone, methadone is used to alleviate severe pain (Argoff and Silvershein, 2009). Despite its considerable therapeutic applications, acute methadone intoxication may lead to morbidity and death (Shields et al., 2007; Soltaninejad et al., 2014). In the United States, opioid drugs were involved in 61% of all drug

overdose deaths and caused more than 28,000 deaths in 2014 (Rudd et al., 2016). Acute poisoning with methadone continues to occur after therapeutic, recreational or accidental use (Jones et al., 2012). In Iran, opium was the drug of choice in 50% of all drug abuse from 2006 to 2009, but the prevalence of methadone toxicity has increased significantly from 2.26% in 2006 to 24.72% in 2011 (Hassanian-Moghaddam et al., 2014).

Since the opiate naïve patients have no tolerance to opiates, (Drummer et al., 1992; Milroy and Forrest, 2000) the stabilization phase should be carefully assessed to reduce the risk of overdose during the induction period to avert the risk of toxicity and death in methadone maintenance treatment (MMT) programs (Morgan et al., 2006; Modesto-Lowe et al., 2010). Several studies indicate a 10-fold increase in methadone-induced toxicity and related death after the increase in the number of methadone maintenance clinics and its arbitrary consumption in recent decades (Shields et al., 2007; Graham et al., 2008). The incidence of poisoning with methadone in children is common due to the availability of this drug as used by family members (Pragst et al., 2013; Shadnia et al., 2013). Methadone poisoning should be considered as a serious threat to naïve, healthy subjects, especially children, as very low doses can cause severe complications or death due to its toxicity (Modesto-Lowe et al., 2010; Jabbehdari et al., 2013; Hassanian-Moghaddam et al., 2017). Indeed, some studies have associated therapeutic doses of methadone with the occurrence of sudden death due to respiratory apnea or cardiac arrest (Chugh et al., 2008).

Few studies have examined cognitive and sensorimotor performance after an acute dose of methadone-induced toxicity in clinical or experimental trials in healthy volunteers. Most studies have examined the effect of the prolonged use of methadone, which can result in neuropsychological impairment as compared to opioid-naïve, healthy controls (Prosser et al., 2006). There is considerable evidence that chronic exposure to methadone in animals can have an adverse effect on memory processes (Hepner et al., 2002; Verdejo et al., 2005). Moreover, patients undergoing the MMT program usually experience limited short-term memory and deficits in working memory (Sjøgren et al., 2000; Mintzer and Stitzer, 2002), visuospatial attention, long-term memory (Prosser et al., 2006) and general cognitive speed (Mintzer et al., 2005) which are in part due to white matter abnormalities (Lin et al., 2012). It has been shown that acute administration of methadone impairs sensorimotor abilities and memory retrieval in rats (Tramullas et al., 2007). Because it has a significantly long half-life of 25–52 h, even a single acute administration of methadone can cause delayed clinical manifestations, including respiratory depression, apnea and unexpected death (LoVecchio et al., 2007).

Despite the fact that, in recent years, methadone overdose has increased, little data is available about the adverse manifestations of methadone overdose in experimentally naïve animals. In addition, behavioral research in human subjects is extremely rare because of ethical considerations. The present study aimed to investigate whether or not a single toxic dose of methadone will result in apnea-caused impairment on cognitive and/or motor functions in adolescent rats.

MATERIALS AND METHODS

Animal

One-month- old male Wistar rats, (Pasteur Institute, Tehran, Iran) weighting 50-80 g, were kept under the standard laboratory conditions (22°C, 12-h light/12-h dark cycle) and randomly allocated to different experimental groups. All rats habituated to their new environment for 5 days before the experimental procedure started. The tests were performed between 8:00 and 16:00 h. All procedures were conducted according to the Guide for the care and use of laboratory animals (National Institutes of Health Publication No. 80–23, revised 1996) and were approved by the Research and Ethics Committee of School of Medicine, Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1395.33), Tehran, Iran.

Drugs

In the present study, Methadone hydrochloride 5 mg/ml (Darou-Pakhsh Pharmaceutical Company, Tehran, Iran) and Naloxone 0.4 mg/ml (Tolid-Darou Pharmaceutical Company, Tehran, Iran) were used.

Experimental Design and Drug Administration

In order to induce acute methadone overdose, rats intraperitoneally (i.p.) received a single toxic dose of 15 mg/kg of methadone at equivalent doses (80% of the LD50) which was chosen based on Chevillard study (Chevillard et al., 2009). Slow and difficult breathing, dizziness, cold and clammy skin, motionlessness, drowsiness, straub tail, muscular rigidity, plantar cyanosis, and irritability were seen after the administration of a single toxic dose of methadone in adolescent rats. It has been noted that the primary signs of opioid intoxication include: pinpoint pupils, respiratory depression, and confusion/unconsciousness, referred to as the opioid overdose triad (Ford, 2001). In 35% of all rats, methadone- induced apnea and caused death if they were left untreated, but the rest of the animals regained normal respiration rate after a few hours without any intervention which was randomly selected as methadone group. In order to evaluate cognitive and motor functions in the rats which experienced apnea (cessation of respiration was for 20 s) (Gaspari and Paydarfar, 2007), an acute single dose of naloxone (2 mg/kg; i.p.) (Farahmandfar et al., 2010; Zamani et al., 2015) was administered following methadone-induced apnea. In addition, resuscitation procedure was performed by a respirator pump to serve artificial respiration (Model V5KG, Narco-Biosystems Inc., Houston, TX, United States). Naloxone administration which was done concomitantly with resuscitation, recovered apnea in 67% of all rats in which had cessation of respiration for 20 s. Therefore, animals which have been successfully resuscitated, were randomly divided into three groups so-called "M/N" groups (rats which received naloxone after methadone overdose) to measure neurological functions either on day 1st, 5th, or 10th post- resuscitation (including M/N-Day 1, M/N-Day 5, and M/N-Day 10 groups; **Figure 1**). In other groups all behavioral

tests were carried out only 1 day after the drug administration. Methadone group was selected randomly from the animals which re-obtained normal respiration rate after administration of a single toxic dose of methadone without any intervention. There was another group without apnea (M/N-Sedate) in which they received the same dose of methadone but naloxone administration was delivered at the beginning of the sedation state. Naloxone group with a single administration dose was designated to test memory and motor functions exclusively in a separated group. Control (Saline) group received an equal volume of saline 0.9% and behavioral assessment was performed 1 day after saline injection. It has been noted that data in the M/N-Day 1, 5 and 10 groups were compared with the Saline control-Day 1, Day 5 and Day 10 groups, respectively. Each group consisted of 6–14 rats while were grouped in 10 experimental groups.

Behavioral Training

For five consecutive days, rats were handled for 5 min before starting any test procedure. All rats had multiple behavioral tests including the Y-maze, novel object memory recognition (NOR) tests as well as pole, rotarod tasks to investigate neurological functions. Animal behaviors were observed by a researcher who was blind to the experimental groups. The order of tests was the same for all animals. In order to avoid the effect of any confounding factors or minimize the influence of stress on animals for each task, the order of behavioral tests was as follow; (1) Y-maze test, (2) NOR test, (3) pole test, and (4) rotarod test. In addition, locomotor activity was measured for each rat during a 5-min period on the test day (**Figure 6**).

Spontaneous Alternation Behavior Test (Y-Maze)

The Y-maze test can be used as a measure of spatial working memory in rodents. It is applied to evaluate the natural tendency of animals to explore new places by recording spontaneous alternation behavior. In this study, the Y-maze apparatus consisted of the Y-shaped maze with three identical arms at 120 degrees to each other which was made of gray- painted Plexiglass. Rats were placed at the end of the one arm and allowed to navigate the maze during an 8-min trial. The sequence and number of the total arm entries were manually registered. An arm entry was defined when four paws were within the arm. An alternation behavior was determined from consecutive entries into the three different arms. The percentage of alternation was calculated as the following equation:

$$\{(\text{number of alternation})/(\text{total number of arm entries} - 2)\} \times 100$$

Total number of arm entries were recorded as well. In addition, animals with 8 arm entries or less were omitted from analysis during an 8-min session (Holcomb et al., 1998; Ma et al., 2007; Farhadinasab et al., 2009).

Novel Object Recognition Test (NOR)

The task procedure consisted of three distinct phases: habituation, familiarization, and test. The NOR task was performed to measure non-spatial memory. An open field box (40 × 40 × 40 cm) (length × width × height) was made of black wood used as an apparatus to test recognition memory. Rats were allowed 1 h of accustomed to the test room before starting each phase. All rats were given a 10-min session to explore apparatus with no objects as a habituation phase in two consecutive days. During the familiarization phase, two identical objects (A1 and A2) were attached to the floor at an equal distance, 10 cm from the walls while positioned in the two adjacent corners. Each rat was placed in the box facing the wall opposite the two identical objects allowed to explore freely for 3 min. If the total exploration time was less than 12 s for the novel and familiar objects during familiarization phase, the rat excluded from the data analysis. Then, object A1 or A2 was replaced with object B before starting the test phase. Evaluation of short-term memory was conducted 90-min later in which the familiar object and the new object (object B) located in the open field. Rats were allowed to explore freely for 3-min in the box. After 24 h, object B was replaced with object C for testing long-term memory in a 3-min period to explore the box. The time spent exploring both objects (familiar and novel) was recorded by a video tracking system. The preference index was calculated as the exploration time for the novel and familiar objects relative to the total time (Antunes and Biala, 2012; Cohen and Stackman, 2015).

Pole Test

Pole test was first introduced by Ogawa (Ogawa et al., 1985) to evaluate movement impairment and coordination in mice indicating a practical task for the basal ganglia dysfunction. The apparatus consisted of a 90 cm vertical wooden pole length and 5 cm in diameter which covered with the rough surface that led into their home cage. All animals received training sessions on two consecutive days (10-trials/day) before the test day where they were placed with the head facing upward right below the top. During the first trial on the first day, if the rat failed to climb down, it was gently turned around on the pole and thus forced to return to its home cage. On the test day, three parameters were measured; t-turn (the time to turn downward), descending time (the time to descend the pole) and total time (the time to turn downward and descent the pole to reach the floor). When the animal failed to turn downward after 120 s, it was taken as a default value. The animals were tested on 3 trials on the test day and the average time was used as the pole test score.

Rotarod Test

Rotarod apparatus is used to evaluate motor coordination and skills in rodents (Dunham and Miya, 1957; Deacon, 2013). Animals were placed on a 2.5 cm diameter drum supported 25 cm above the base of the apparatus. Rats were trained 5 trials a day, separated by 30 min inter-trial intervals on the two successive days. Animals were placed in the testing room for 1 h before starting the test to acclimate to the testing. The rats were held by their tails while facing away from the direction of rotation

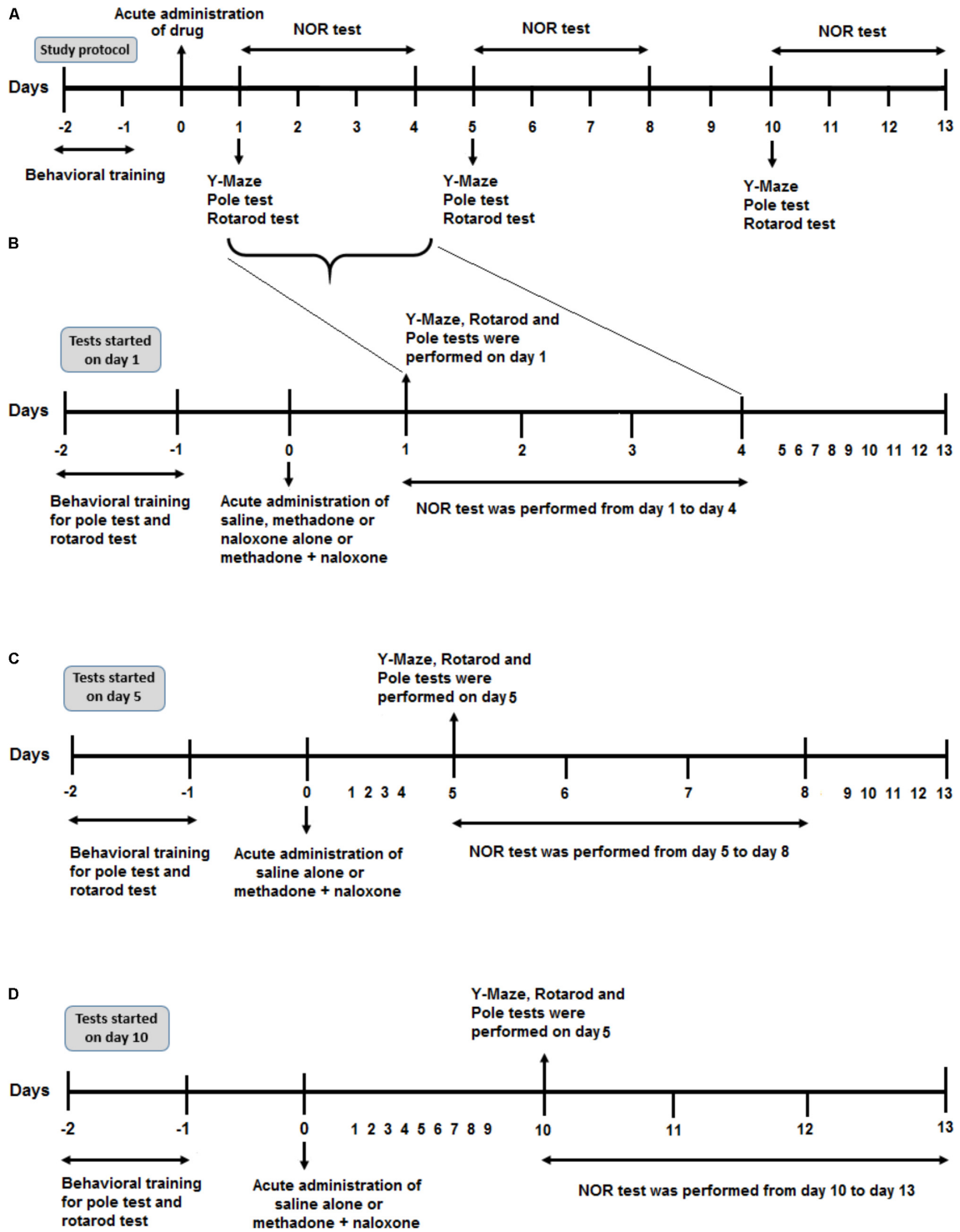


FIGURE 1 | Schematic illustration of the experimental schedule. **(A)** Protocol overview of the study. After 2 days of behavioral training (rotarod and pole tasks), all rats were administered drug injection on day 0. According to the time of the behavioral test, (starting on day 1, 5, or day 10 after drug administration) they represented here in three parts. **(B)** After drug application, six separated animal groups (Saline, Methadone, Naloxone, M/N-Sedate, Saline control-Day 1, and M/N-Day 1) were used to evaluate different behavioral tests including Y-Maze, Rotarod test and Pole test on day 1 after drug administration and Novel object recognition (NOR) test from day 1 to day 4 post-treatment. **(C)** In two other separated groups (Saline control-Day 5, M/N-Day 5), after administration of saline alone or methadone + naloxone on day 0, Y-Maze, Pole test, and Rotarod test were carried out on day 5 followed by NOR test from day 5 to day 8. **(D)** In Saline control-Day 10 and M/N-Day 10 groups, 10 days after administration of saline alone or methadone + naloxone, Y-Maze, Pole test and Rotarod test were carried out on day 10 followed by NOR test from day 10 to day 13.

the drum such that animals released on the horizontal rod while walking forward to keep their balance. The Rotation speed was set at 20 rpm in the training and testing sessions. If the rat failed to grasp rod properly and fell before 5 s, the procedure would start again to keep the balance. During the test session, animals were assessed by placing on the rod until either they fell off or reached a maximum 300 s. The mean values of the 3 test trials were calculated for each rat.

Locomotor Activity

Total numbers of infrared beam break automatically were recorded. Rats were placed in a box (40 × 40 × 40 cm) to evaluate locomotion. Locomotor activity was tracked by a 5 × 5 photobeam configuration for each rat in which sensed infrared beam interruption caused by movement of the animal in real time for 5 min (Zhang and Kong, 2017).

Statistical Analysis

All data were represented as mean ± SEM (standard error of mean) and were analyzed by commercially available software GraphPad Prism® 5.0. In order to compare data between two groups in familiarization phase in NOR, data in apnea groups which have been compared with their respective Saline control groups, paired or unpaired *t*-test were used, respectively. For multiple comparisons between groups, one-way analysis of variance (ANOVA) followed by *post hoc* Newman–Keuls test was applied as needed. The level of statistical significance was set at *P*-value less than 0.05 ($P < 0.05$).

RESULTS

Effect of a Single Acute Toxic Dose of Methadone Administration on Spatial Working Memory in the Y-Maze Test

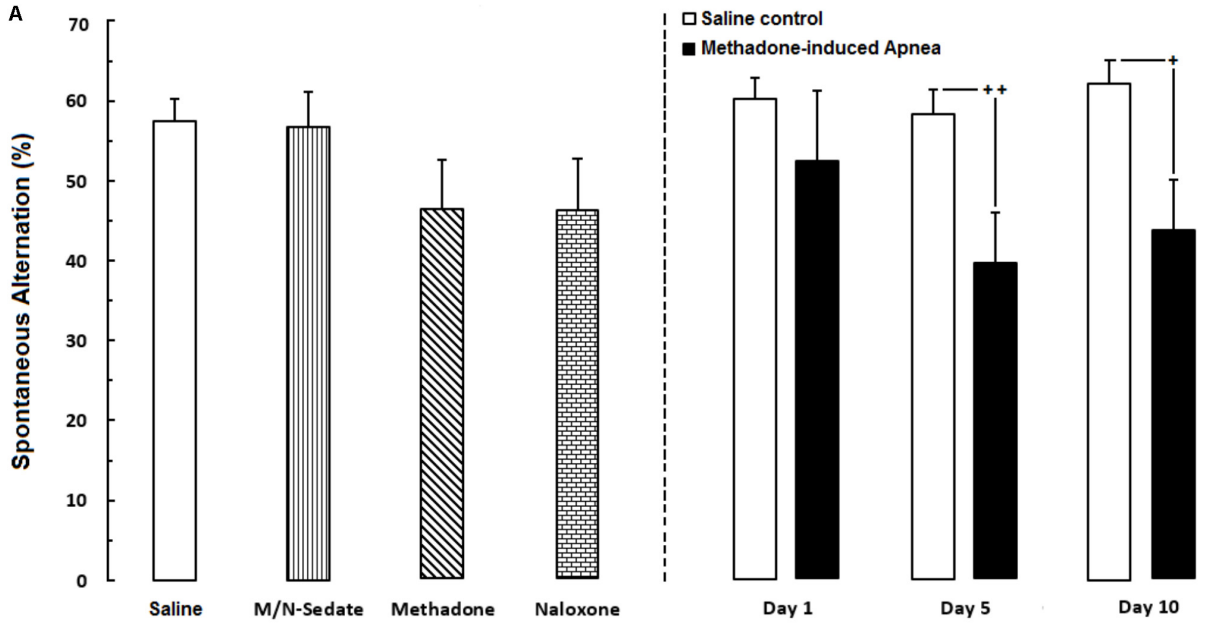
As shown in **Figure 2A**, unpaired *t*-test analysis revealed that administration of an acute toxic dose of methadone (15 mg/kg; i.p.) which caused apnea and subsequent naloxone injection (2 mg/kg; i.p.), impaired the percentage of the spontaneous alternation behavior in the M/N-Day 5 [$t(9) = 2.908$, $P < 0.01$] and M/N-Day 10 groups [$t(10) = 2.695$, $P = 0.0225$] when compared with their respective Saline control groups (right panel) while this parameter was not different in the M/N-Day 1 as compared to its Saline control group [$t(12) = 0.9745$, $P = 0.3491$; ns]. As depicted in **Figure 2A**, one way ANOVA followed by Newman–Keuls *post hoc* analysis showed that there was not significant deficient in the alternation behavior in M/N-Sedate, M/N-treated rats (with apnea, right panel), methadone (without apnea) or naloxone groups as compared with the Saline [$F_{(6,46)} = 1.571$, $P = 0.1773$] group. Moreover, in **Figure 2B**, unpaired *t*-test analysis manifested that the number of arm entries were not different in the M/N-Day 1 [$t(12) = 1.947$, $P = 0.0754$; ns], Day 5 [$t(9) = 0.8357$, $P = 0.4249$; ns] and Day 10 [$t(10) = 1.010$, $P = 0.3363$; ns] groups as compared with their respective Saline control groups (right panel). In addition, One-way ANOVA followed by Newman–Keuls *post hoc* analysis revealed no significant reduction in the number of total arm entries in the M/N-Sedate, as well as M/N-Day 1 and Day 10 groups (with apnea, right panel), methadone or naloxone groups when compared with the Saline group [$F_{(6,47)} = 2.232$, $P < 0.05$] but not for the M/N-Day 5 groups which showed significant reduction as compared with the Saline group.

Effect of an Acute Toxic Dose of Methadone Administration in Recognition Memory in Adolescent Rats

The novel object recognition task is the ability to distinguish the novel from familiar stimuli which is directly dependent on the prefrontal cortex and hippocampus function (Banks et al., 2012; Pezze et al., 2017). In **Figure 3A**, the data obtained analyzed using paired *t*-test exhibited that animals spent equal time to explore both object A1 and A2 and there were not any significant preference in exploring two objects in familiarization phase in the M/N-Day 1 [$t(6) = 0.7381$, $P = 0.4883$; ns], Day 5 [$t(6) = 0.5558$, $P = 0.5984$; ns] and Day 10 [$t(6) = 0.5176$, $P = 0.6109$; ns] groups as compared with their respective Saline control groups (right panel). In **Figure 3B** which shown short-term memory phase, unpaired *t*-test analysis indicated that a single toxic dose of methadone (apnea groups) significantly impaired recognition memory in the M/N-Day 1 [$t(12) = 2.785$, $P < 0.01$] and M/N-Day 5 [$t(9) = 3.032$, $P < 0.01$] when compared with their respective Saline control groups (right panel). One-way ANOVA followed by Newman–Keuls *post hoc* test exhibited that administration of an acute toxic dose of methadone (15 mg/kg; i.p.) with subsequent naloxone (2 mg/kg; i.p.) administration in sedation state (M/N-Sedate group), three M/N-treated groups, as well as methadone and naloxone groups did not have attenuating effects on short-term memory when compared to the Saline group [$F_{(6,46)} = 3.871$, $P = 0.0010$], while this parameter shown significant reduction in the M/N-Day 5 groups as compared with the Saline group. In **Figure 3C**, the data obtained for long-term memory test revealed detrimental effect of methadone overdose on long-term memory in the M/N-Day 1 [$t(11) = 3.903$, $P = 0.0025$] and Day-5 [$t(9) = 4.512$, $P < 0.001$] groups which have been continued on day 13 in M/N-Day 10 group [$t(11) = 4.285$, $P < 0.001$] when compared with their respective Saline control groups (right panel). Moreover, administration of an acute toxic dose of methadone (15 mg/kg; i.p.) or naloxone (2 mg/kg; i.p.) alone as well as M/N-treated rats (right panel) and M/N-Sedate groups did not show any significant deficit in long-term memory when compared to the Saline group [$F_{(6,46)} = 2.384$, $P = 0.0434$].

Effect of an Acute Toxic Dose of Methadone Administration on Motor Functions in Pole Test in Adolescent Rats

As exhibited in **Figure 4A** (right panel), unpaired *t*-test analysis showed that there was no significant difference in t-turn between the M/N-Day 1 [$t(18) = 1.674$, $P = 0.1115$; ns], Day 5 [$t(10) = 1.820$, $P = 0.0988$; ns] and their respective Saline control groups but this parameter increased in the M/N-Day 10 group as compared with its respective Saline control group [$t(12) = 2.180$,



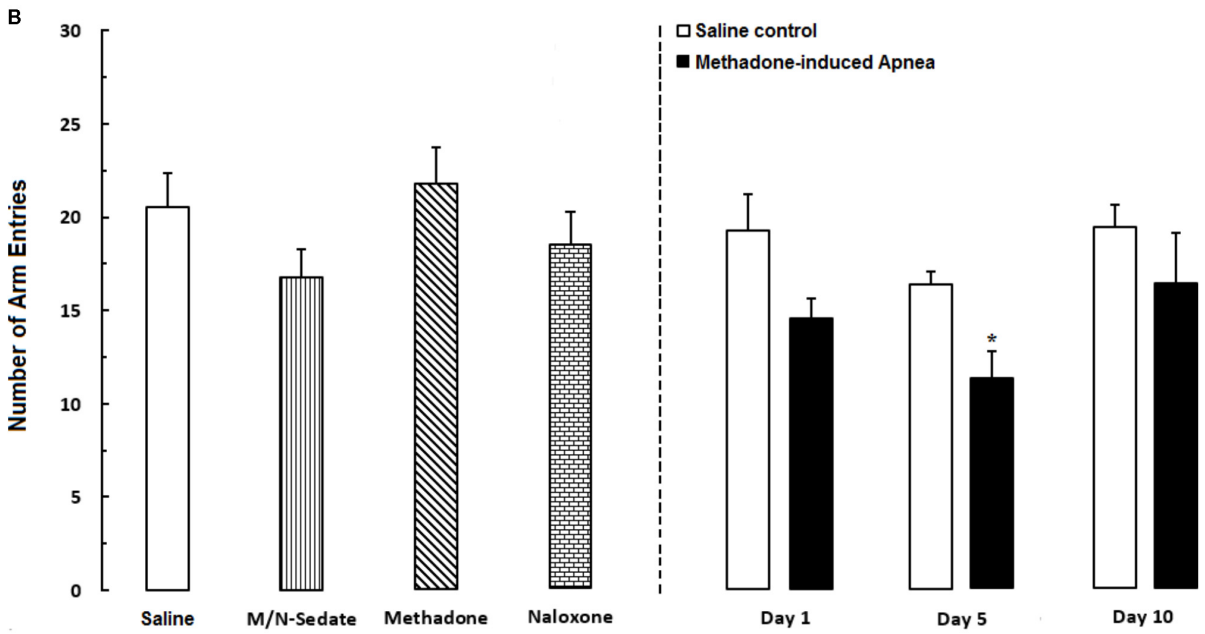


FIGURE 2 | (A) Spontaneous alternation behavior and **(B)** Total number of arm entries were recorded in different groups, including; M/N-treated groups (a single dose of naloxone was administered after methadone overdose in apnea stage, in animals which experienced apnea and spontaneous alternation behavior was evaluated either on day 1, 5, or 10 day post-resuscitation; M/N-Day 1 (n = 6), M/N-Day 5 (n = 6) and M/N-Day 10 (n = 6) groups and their respective Saline control groups; n = 6 right panel), M/N-Sedate (n = 8; a single dose of naloxone was administered following methadone overdose, immediately in the initial stage of sedation, so behavioral evaluation was carried out only 1 day after drug administration), Saline; n = 12, methadone; n = 8 and naloxone; n = 7 (saline, methadone or naloxone were administered alone in separated groups in which spontaneous alternation behavior and the number of arm entries were recorded only 1 day after drug administration during an 8-min trial in adolescent rats) groups. Animals received methadone (15 mg/kg; i.p.) or naloxone (2 mg/kg; i.p.) alone or both (apnea groups) in a single dose. Each bar shows the mean ± SEM for 6–12. $^{*}P < 0.05$ different from the Saline group. $^{+}P < 0.05$ and $^{++}P < 0.01$ different from their respective Saline control groups.

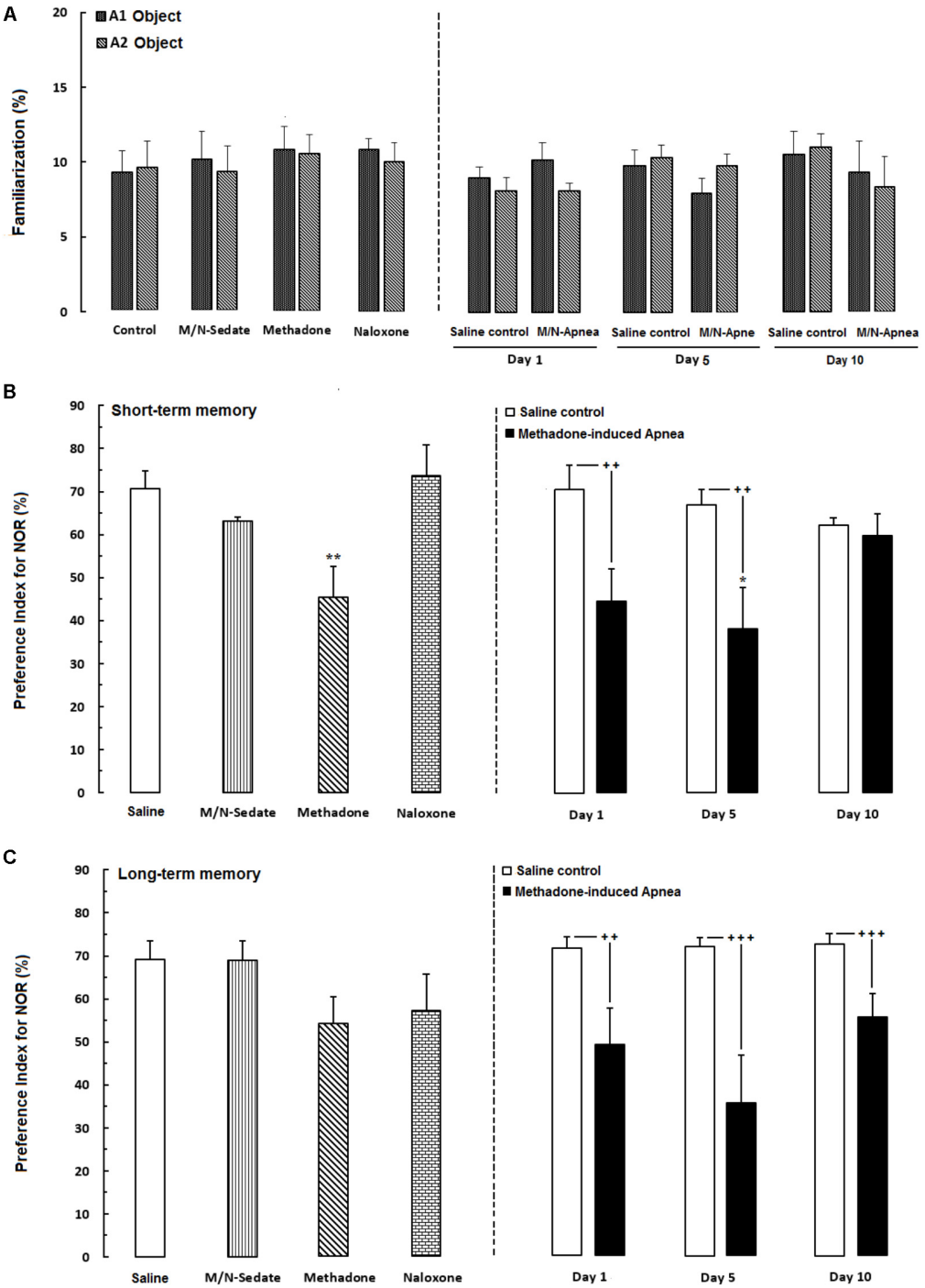


FIGURE 3 | Performance of recognition memory in the novel object recognition task in three sessions as follow; **(A)** Familiarization (rats were allowed to explore freely two identical objects A1 and A2 for 3-min), **(B)** Short-term memory (object A1 or A2 was replaced with object B while rats were allowed to explore for 3-min), **(C)** long-term memory (object B was replaced with object C which provided rats explored freely two objects for 3-min) in different groups, including; M/N-treated groups (a single dose of naloxone was administered after methadone overdose in apnea stage, in animals which experienced apnea and recognition memory was evaluated either on day 1, 5, or 10 day post-resuscitation; M/N-Day 1 (n = 7), M/N-Day 5 (n = 6) and M/N-Day 10 (n = 6) groups and their respective Saline control groups (n = 6); right panel), M/N-Sedate (n = 7; a single dose of naloxone was administered following methadone overdose, immediately in the initial stage of sedation, so behavioral evaluation was carried out only 1 day after drug administration), Saline; n = 10, methadone; n = 11, naloxone; n = 8 (Saline, methadone or naloxone were administered alone in separated groups which recognition memory was evaluated only 1 day after the drug administration in adolescent rats) groups. Animals received methadone (15 mg/kg; i.p.) or naloxone (2 mg/kg; i.p.) alone or both in a single dose. Each bar shows the mean ± SEM for 6–11. $^{*}P < 0.05$ and $^{**}P < 0.01$ different from the Saline group. $^{++}P < 0.01$ and $^{+++}P < 0.001$ different from their respective Saline control groups.

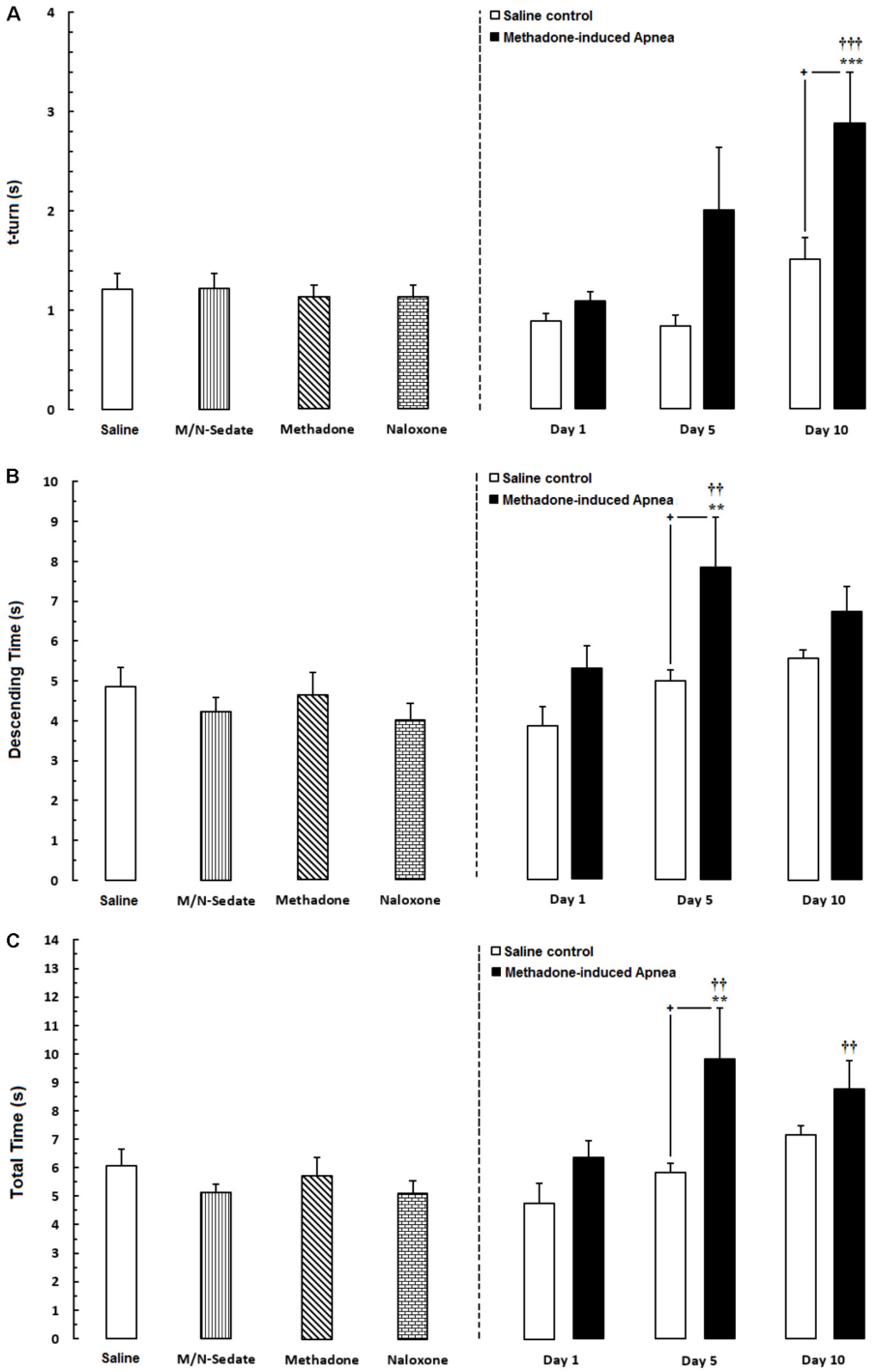


FIGURE 4 | Evaluation of motor performance in pole test such that three parameters were measured including; **(A)** time to turn downward (t-turn), **(B)** descending time (time to move downward to reach the floor and **(C)** total time (time to turn and descending the pole to reach the floor) in different groups, including; M/N-treated groups (a single dose of naloxone was administered after methadone overdose in apnea stage, in animals which experienced apnea and motor behavior was evaluated either on day 1, 5, or 10 day post-resuscitation; M/N-Day 1 (n = 11), M/N-Day 5 (n = 6) and M/N-Day 10 (n = 7) groups and their respective Saline control groups; n = 6; right panel), M/N-Sedate; n = 9 (a single dose of naloxone was administered following methadone overdose, immediately in the initial stage of sedation, so behavioral evaluation was carried out only 1 day after drug administration), Saline; n = 14, methadone; n = 8 and naloxone; n = 8 (saline, methadone or naloxone were administered alone in separated groups in which motor function was evaluated 1 day after drug administration during pole test in adolescent rats) groups. Animals received methadone (15 mg/kg; i.p.) or naloxone (2 mg/kg; i.p.) alone or both in a single dose. Each bar shows the mean ± SEM for 6-14. $^{**}P < 0.01$ and $^{***}P < 0.001$ different from the Saline group. $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ different from the M/N-Sedate group. $^{+}P < 0.05$ different from their respective Saline control groups.

$P < 0.05$]. Moreover, one-way ANOVA followed by Newman–Keuls *post hoc* test showed that in the M/N-Sedate, methadone and naloxone groups no change has been observed in *t*-turn values when compared with the Saline group [$F_{(6,57)} = 6.007$, $P < 0.0001$] but significant increase revealed in the M/N-Day 10 when compared with the Saline or the M/N-Sedate groups. In **Figure 4B** (right panel), unpaired *t*-test analysis indicated that there was no significant difference in descending time in the M/N-Day 1 [$t(18) = 1.793$, $P = 0.0898$; ns] and Day 10 [$t(11) = 1.442$, $P = 0.1772$; ns] groups as compared with their respective Saline control groups, but in the M/N-Day 5 group, the impairment was obvious in descending time when compared with its respective Saline control group [$t(10) = 2.209$, $P < 0.05$]. However, as shown in **Figure 4B**, one way ANOVA revealed that in the M/N-Sedate, the M/N-Day 1 and Day 10 groups as well as methadone (without apnea) and naloxone groups, motor functions were not impaired as compared with the Saline group [$F_{(6,56)} = 4.221$, $P = 0.0014$] but significant increase in descending time was observed in the M/N-Day 5 when compared with the Saline or M/N-Sedate groups. Additionally, in **Figure 4C** (right panel), unpaired *t*-test analysis showed that there was no significant impairment in motor function in the M/N-Day 1 [$t(18) = 2.008$, $P = 0.0599$; ns] and Day 10 [$t(12) = 1.307$, $P = 0.2155$; ns] groups as compared with their respective Saline control groups, but in the M/N-Day 5, the detrimental effect of methadone overdose was seen in motor activity when compared with its respective Saline control group [$t(10) = 2.217$, $P < 0.05$]. Furthermore, As depicted in **Figure 3C**, One-way ANOVA followed by Newman–Keuls *post hoc* test revealed that motor function did not impair in the M/N-Sedate as well as in methadone and naloxone groups as compared with the Saline group [$F_{(6,56)} = 4.605$, $P = 0.0007$] but total time increased significantly in the M/N-Day 5 and Day 10 groups in comparison with the Saline or M/N-Sedate groups.

Rotarod Test

Unpaired *t*-test analysis indicated that there were no significant detrimental effect of methadone overdose on motor coordination in rotarod test in the M/N-Day 1 [$t(14) = 1.915$, $P = 0.0762$; ns], Day 5 [$t(11) = 2.153$, $P = 0.0543$; ns] and Day 10 [$t(13) = 1.689$, $P = 0.1150$; ns] groups when compared with their respective Saline control groups (right panel). In addition, One-way ANOVA with Newman–Keuls *post hoc* test showed that in methadone group (without apnea) there was significant impairment in motor coordination as compared with the Saline group [$F_{(6,49)} = 3.386$, $P = 0.0071$]. However, in M/N-Sedate and naloxone groups as well as three M/N-treated groups, no significant differences revealed on motor coordination when compared with the Saline group (**Figure 5**).

Effect of Acute Toxic Dose of Methadone on Locomotor Activity

As shown in **Figure 6**, unpaired *t*-test analysis showed that a single dose of naloxone (2 mg/kg; i.p.) administration following methadone overdose (15 mg/kg; i.p.) in animals which experienced apnea did not displayed significant deficit in locomotor activity in the M/N-Day 1 [$t(15) = 0.3083$, $P = 0.7621$; ns], Day 5 [$t(11) = 0.6218$, $P = 0.5468$; ns] and Day 10 [$t(10) = 0.0126$, $P = 0.9902$; ns] groups when compared to their respective Saline control groups (right panel). In addition, One-way ANOVA by Newman–Keuls *post hoc* analysis showed that there was no significant difference in locomotor activity in the M/N-Sedate group, M/N-treated rats (right panel, with apnea) as well as methadone and naloxone groups when compared with the Saline group [$F_{(6,62)} = 1.084$, $P = 0.3817$].

DISCUSSION

The purpose of this research was to investigate the cognitive and motor effects of a single toxic dose of methadone on three random groups of naïve adolescent rats tested on either day 1, 5, and 10 after drug administration as depicted in **Figure 1**. The findings showed that (i) Administration of an acutely toxic dose of methadone induced apnea in 35% of treated rats, (ii) Naloxone as a non-specific opioid receptor antagonist resuscitated 67% of the rats which experienced apnea, (iii) Delay-dependent impairment in cognitive and motor functions was observed in different behavioral tests, (iv) Transient motor impairment following methadone-induced apnea, and (v) Motor deficient in descending time on day 5 after administration of an acute toxic dose of methadone overdose in pole test was observed.

It has been documented that opioids suppress respiration in humans and animals (Van Der Schier et al., 2014). Methadone is a long-acting opioid agonist used for therapy and as medication for abuse/dependence and to treat severe refractory cancer pain (Leppert, 2009; Keane, 2013; Schuckit, 2016). The extensive prescription of methadone has enhanced the risk of life-threatening overdoses in different countries (Paulozzi et al., 2006; Rudd et al., 2016). Buprenorphine, like methadone, is used in the treatment of opioid addiction, but as a partial agonist, displays a ceiling effect; after a certain point, an increase in the dosage will not enhance its effects (Dahan et al., 2006). In contrast, methadone is a full opioid agonist which has the potential to be abused, misused or used non-medically, making overdose-related death, especially due to respiratory depression, a big problem (Ayatollahi et al., 2011; Whelan and Remski, 2012; Soltaninejad et al., 2014). In recent years, methadone has been extensively prescribed in the MMT programs or to relieve pain, giving rise to methadone overdoses by adults using supratherapeutic amounts or by accidental ingestion in the pediatric population (Soltaninejad et al., 2014).

In the Y-maze task, which is a measure of spatial working memory, impairment was revealed in the alternation behavior after methadone overdose. Consistent with our results, Hepner et al. (2002) indicated that methadone which acutely administered impaired the working memory version of Morris water task in rats. In the current study, we did not measure the concentration of methadone in blood or brain tissues, but Andersen et al. (2011) indicated that no methadone detected in brain tissue on the test day which showed memory impairment after the drug administration. The long-lasting impairment in learning or memory after acute or chronic opioid administration

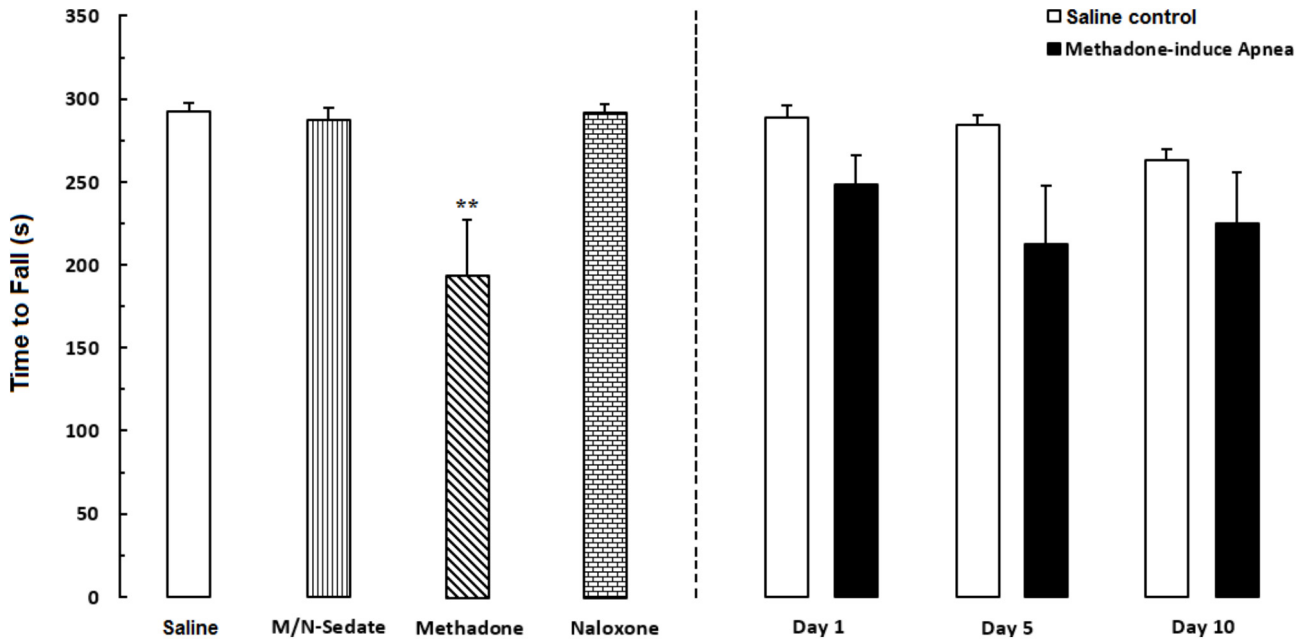


FIGURE 5 | Evaluation of motor coordination and balance in rotarod test during a 5-min trial in different groups, including; M/N-treated groups (a single dose of naloxone was administered after methadone overdose in apnea stage, in animals which experienced apnea and motor coordination was evaluated either on day 1, 5, or 10 day post-resuscitation; M/N-Day 1 (n = 9), M/N-Day 5 (n = 6) and M/N-Day 10 (n = 8) groups and their respective Saline control groups; n = 6; right panel), M/N-Sedate (n = 8; a single dose of naloxone was administered following methadone overdose, immediately in the initial stage of sedation, so behavioral evaluation was carried out only 1 day after drug administration), Saline; n = 10, methadone; n = 9 and naloxone; n = 6 (saline, methadone or naloxone was administered alone in separated groups in which coordination assessment was evaluated only 24 h following the drug administration in a 5-min trial in adolescent rats) groups. Animals received methadone (15 mg/kg; i.p.) or naloxone (2 mg/kg; i.p.) alone or both in a single dose. The time each rat stay on the rod before falling was recorded. Each bar shows the mean ± SEM for 6–10. $^{**}P < 0.01$ different from the Saline group.

might be associated with the persistent impairment of different brain functions through several mechanisms, including changes in central signaling proteins (Lou et al., 1999), activation of apoptosis signaling pathway (Emeterio et al., 2006) or impaired synaptic plasticity (Pu et al., 2002). Previous results suggest that the hippocampus and prefrontal cortex are involved in the NOR task (Banks et al., 2012; Pezze et al., 2017). The current findings indicate impairment of short-term memory in the NOR task on days 3, 7, and 12 (timeline was shown in **Figure 1**) after methadone overdose. In addition, the reduced recognition memory in the methadone (without apnea) group and the M/N groups which experienced apnea might be due to the activation or changes in protein signaling or the apoptosis pathways related to methadone overdose which have been induced by a high-dose application of opioids (Tramullas et al., 2007; Andersen et al., 2012). It has been proposed that memory impairment might be the result of the direct toxicity of methadone that overstimulates the opioid receptors in the hippocampus and limbic system related to particular forms of learning and memory, including spontaneous object recognition memory (Pertschuk and Sher, 1975; Wehner et al., 2000). Recognition impairment has been observed in previous studies that have described damage to the hippocampus as sufficient to create impairment of recognition memory (Broadbent et al., 2010).

Pole test measurements reflected the deterioration of motor function in the M/N-Day 5 but not in the M/N-Day 1 group. In the current study, the transient impairment of motor performance after methadone overdose suggests that perhaps alternative strategies with other brain regions involved in the processing of sensorimotor performance. Another explanation is, administration of naloxone (reversing methadone overdose) may partly reduce the motor disabilities following injection of a toxic dose of methadone with unknown mechanisms.

In the present study, the rotarod test for evaluation of motor coordination and balance showed mild or no deficiency in animals which experienced apnea. It is important to note that the lack of significant impairment in motor performance in the rotarod test could be in part due to the small number of rats which experienced apnea that had executed the test. It should be noted that other conditions such as test protocol, laboratory environmental factors, and rod diameter could have influenced the sensitivity of the test for detecting subtle deficiencies in motor function or balance following methadone-induced apnea. Nevertheless, several previous reports indicated the lack of motor coordination, executive function, and ataxia which were observed following methadone overdose (Tramullas et al., 2007; Cottencin et al., 2009).

The current results showed no changes in locomotor activity after a single toxic dose of methadone, which is inconsistent with the results of previous studies on the attenuating or increasing

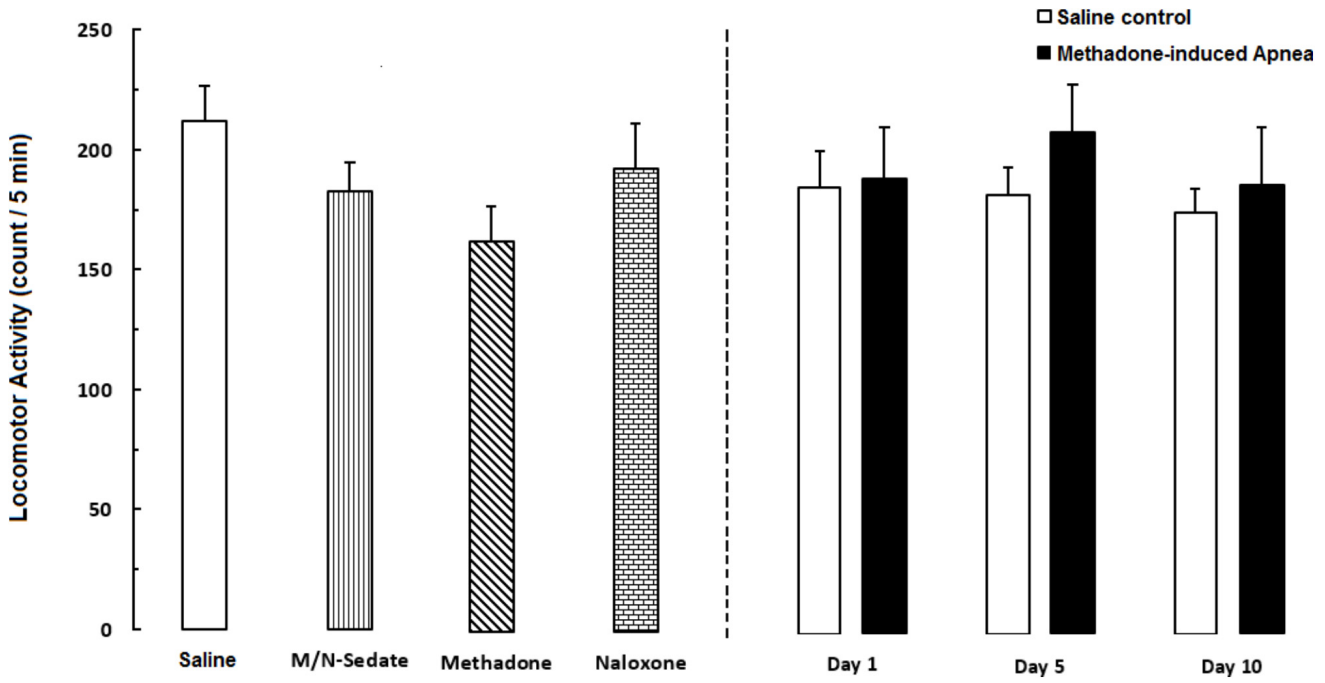


FIGURE 6 | Locomotor activity was recorded by using photobeam activity system during a 5-min session in different groups, including; M/N-treated groups (a single dose of naloxone was administered after methadone overdose in apnea stage, in animals which experienced apnea and locomotor activity was evaluated either on day 1, 5, or 10 day post-resuscitation; M/N-Day 1 (n = 10), M/N-Day 5 (n = 6) and M/N-Day 10 (n = 6) groups and their respective Saline control groups; n = 6; right panel), M/N-Sedate (n = 11; a single dose of naloxone was administered after methadone overdose immediately in the initial stage of sedation state, so behavioral evaluation was carried out only 1 day after drug administration), Saline; n = 14, methadone; n = 12 and naloxone; n = 8 (saline, methadone or naloxone was administered alone in separated groups in which locomotor activity was evaluated 1 day after drug administration) groups. Animals received methadone (15 mg/kg; i.p.) or naloxone (2 mg/kg; i.p.) alone or both in a single dose. Administration of toxic dose of methadone did not change locomotor activity in all groups. Each bar shows the mean ± SEM for 6–14.

effect of motor activity following acute or chronic administration of methadone in rats (Mendez and Trujillo, 2008). Different routes of administration, patterns and doses of methadone prescribed, and the duration of recording of locomotion, as well as the different time point measurements, might affect the outcomes and produce different results (Allouche et al., 2013).

It also has been suggested that administration of naloxone after methadone overdose may modulate the detrimental effects of opioid receptor activation on both locomotor activity and motor coordination in the rotarod task. The results showed that administration of a single dose of naloxone had no effect on memory or motor performance. Hayward and Low reported that naloxone dose-dependently decreased motor activity, which is inconsistent with the current findings (Hayward and Low, 2005). It appears that the short duration of action of naloxone (Aghabiklooei et al., 2013) did not cause alterations in motor function at 24 h post-injection.

Administration of NMDA receptor antagonists like AP5 and MK-801 could impair spatial working memory. As a result, the antagonistic action of methadone on the NMDA receptors might confirm the hypothesis that methadone mediates through both opioid and NMDA receptors to exert adverse/neurotoxic effects on memory and motor function in different behavioral tasks (Ebert et al., 1995; Tsien et al., 1996; Nylander et al., 2016).

Delayed leukoencephalopathy was described for the first time after anoxic injury with symmetrical necrotic lesions of the central white matter, along with the damage to gray matter caused delayed neurological deterioration after initial recovery (Lin et al., 2012; Meyer, 2013). There are several reports of severe methadone-induced leukoencephalopathy which can be recognized by magnetic resonance imaging findings (Mittal et al., 2010; Cerase et al., 2011). The exact mechanism remains uncertain, but one possible hypothesis is that it is in part due to the defect in energy metabolism caused by demyelination following respiratory depression/arrest after methadone overdose (Weinberger et al., 1994). Direct damage or activation of immunological responses to brain tissue is another hypothesis which explains the pathogenesis of methadone-induced leukoencephalopathy (Mills et al., 2008; Mittal et al., 2010; Cerase et al., 2011; Rando et al., 2016). The serum half-life of morphine administered to an opioid-naïve patient was nearly 2–3 h, while this for methadone is approximately 150 h (Ciejka et al., 2016). With respect to methadone toxicity in cell culture, it has been suggested that methadone-induced cell death uncouples mitochondria, resulting in impairment of ATP synthesis (Perez-Alvarez et al., 2010;

Nylander et al., 2016). Although these findings are not specific, such symptoms are in part consistent with the current behavioral results. Acute cerebellitis (Mills et al., 2008; Rando et al., 2016) or basal ganglia (Cottencin et al., 2009; Corliss et al., 2013) damage involvement following methadone overdose may explain the motor impairment observed in the pole and rotarod tasks caused in part by overstimulation of opioid receptors in these brain regions.

Despite its limitation, our findings indicate that following acute methadone overdose, reporting and follow-up assessment with the use of brain-imaging techniques after relative initial recovery should be performed. Utilization of plausible neurotoxicity biomarkers would allow continual monitoring to explore complications and possible damage to the central nervous system as well. It is suggested that methadone overdose should be considered to be a possible cause of delayed neurological disorders which require accurate monitoring for adverse reactions or signs to aid diagnosis of the risk of complications to the nervous system in hospital poison centers for healthy subjects, especially children. It has been noted that respiratory depression should be considered in patients using opioids for the first time, but not in chronic users because they develop a tolerance to opioid drugs. It has been suggested that overdose with long-acting opioids such as methadone by a naïve individual may require a longer observation period in the hospital to reduce delayed complications or possible long-term sequelae.

The important limitation of this study means, it remains unclear whether the impairments relate directly to methadone toxicity or cerebral hypoxia. Moreover, we did not measure the concentration of methadone in blood or brain tissue on test days due to the limited financial resource at the time of doing research.

CONCLUSION

In contrast to the majority of studies on the neurological consequences of MMT patients, the current study has shown that acute exposure to a toxic dose of methadone in naïve healthy rats impaired cognitive and/or motor function. The deficient was reversible in motor function but not for memory during an observation period of nearly 2 weeks. The exact mechanisms remain uncertain, but further studies are required to elucidate the different pathophysiological mechanisms of methadone-induced neurotoxicity.

AUTHOR CONTRIBUTIONS

All authors designed the study, analyzed and interpreted the data, and wrote the paper.

FUNDING

This study was carried out as a part of Ph.D. thesis written by LA-M funded (Grant/Registration no: 70) by the School of Medicine, Shahid Beheshti University of Medical Sciences.

ACKNOWLEDGMENTS

The authors greatly appreciate the Neuroscience Research Center members for the kindly collaboration.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 25 July 2017
doi: 10.3389/fphar.2017.00496

Effect of Cognitive Style on Learning and Retrieval of Navigational Environments

Maddalena Boccia[1,2]*, *Francesca Vecchione*[1], *Laura Piccardi*[2,3] *and Cecilia Guariglia*[1,2]

[1] Department of Psychology, "Sapienza" University of Rome, Rome, Italy, [2] Cognitive and Motor Rehabilitation Unit, Fondazione Santa Lucia (IRCCS), Rome, Italy, [3] Department of Life, Health and Environmental Sciences, L'Aquila University, L'Aquila, Italy

Field independence (FI) has been found to correlate with a wide range of cognitive processes requiring cognitive restructuring. Cognitive restructuring, that is going beyond the information given by the setting, is pivotal in creating stable mental representations of the environment, the so-called "cognitive maps," and it affects visuo-spatial abilities underpinning environmental navigation. Here we evaluated whether FI, by fostering cognitive restructuring of environmental cues on the basis of an internal frame of reference, affects the learning and retrieval of a novel environment. Fifty-four participants were submitted to the Embedded Figure Test (EFT) for assessing their Cognitive Style (CS) and to the Perspective Taking/Spatial Orientation Test (PTSOT) and the Santa Barbara Sense of Direction Scale (SBSOD) for assessing their spatial perspective taking and orientation skills. They were also required to learn a path in a novel, real environment (route learning, RL), to recognize landmarks of this path among distracters (landmark recognition, LR), to order them (landmark ordering, LO) and to draw the learned path on a map (map drawing, MD). Retrieval tasks were performed both immediately after learning (immediate-retrieval) and the day after (24 h-retrieval). Performances on EFT significantly correlated with the time needed to learn the path, with MD (both in the immediate- and in the 24 h- retrievals), results on LR (in 24-retrieval) and performances on PTSOT. Interestingly, we found that gender interacted with CS on RL (time of learning) and MD. Females performed significantly worse than males only if they were classified as FD, but did not differ from males if they were classified as FI. These results suggest that CS affects learning and retrieval of navigational environment, especially when a map-like representation is required. We propose that CS may be pivotal in forming the cognitive map of the environment, likely due to the higher ability of FI individuals in restructuring environmental cues in a global and flexible long-term representation of the environment.

Keywords: field dependence, human navigation, topographical memory, spatial orientation, patial mental representation, egocentric and allocentric coordinates

OPEN ACCESS

Edited by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico

Reviewed by:
Andrea Bosco, Università degli studi di Bari Aldo Moro, Italy
Santiago J. Ballaz, Yachay Tech University, Ecuador

***Correspondence:**
Maddalena Boccia
maddalena.boccia@uniroma1.it

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *29 May 2017*
Accepted: *12 July 2017*
Published: *25 July 2017*

Citation:
Boccia M, Vecchione F, Piccardi L and Guariglia C (2017) Effect of Cognitive Style on Learning and Retrieval of Navigational Environments. Front. Pharmacol. 8:496. doi: 10.3389/fphar.2017.00496

INTRODUCTION

The "environmental space" has been proposed as the portion of the space that can be inspected and learned through considerable movement, that is actually navigating across buildings and neighbors (Wolbers and Wiener, 2014), while the "vista space" concerns the portion of the space that can be visually inspected and learnt from a single location or with little movements

(Wolbers and Wiener, 2014). To successfully orientate within the environmental space, individuals rely on at least two sources of information (Wolbers and Hegarty, 2010). The first one is the online representation of the space, which consists of information about the current position and its spatial updating, the egocentric self-to-objects relations and distances, the allocentric relations among environmental objects and the route progression. The second one is the offline representation of the environment, which includes the topographical knowledge that allows for building up a stable internal representation of the environmental space, namely the "cognitive map" (Tolman, 1948). Thanks to the topographical knowledge, individuals can imagine what lies beyond the current vista space and, thus, are able to successfully plan the best route toward their navigational goal.

Three different types of mental representations of the environment, namely the Landmark, Route and Survey representations, have been described (Siegel and White, 1975). *Landmark representation* roughly corresponds to the figurative memory of environmental objects; by using this type of knowledge, individuals are able to "beacon" toward salient landmarks within the vista space. *Route representation* consists of the memory of the path that connects different landmarks in the environmental space, organized on the basis of an egocentric frame of reference; by using this type of knowledge individuals are able to reach a not visible navigational goal by recalling the sequence of landmarks, directions and distances along the path connecting the starting point and the goal. *Survey representation* roughly corresponds to a map-like representation of the environmental space, which implies the encoding of directions and distances between landmarks regardless of the individual's position, that is the use of an allocentric frame of reference; by using this type of knowledge individuals are able to reach a navigational goal planning, novel routes and detours. The organization of topographical knowledge across the three mental representations of the environmental space is cumulative, and hierarchical, with high-level stages encompassing features of the lower stages and being mandatory the acquisition of lower-level stages for higher-level stages (Siegel and White, 1975). Interestingly, the functional activation within the brain network involved in spatial navigation, specifically the retrosplenial cortex (RSC) and parahippocampal gyrus (PHG) (see Boccia et al., 2014 for a review), shows an interaction with the acquisition stage of the topographical knowledge and its format. In particular, both RSC and PHG are activated by the visual scanning of the vista space during the first stage of the acquisition, but also by the mental representation of the position of a non-visible landmark when topographical knowledge has been fully acquired (i.e., after 5 days of spatial training; Boccia et al., 2016a). This is consistent with the idea that well-known environments are represented in a survey format allowing to take into account portions of the environment that are not in the direct view (Siegel and White, 1975).

Levels of navigational skills are greatly variable in humankind and this variability has been shown to correspond to neuroanatomical differences, since *good* navigators show higher gray matter volume in the right hippocampus (Wegman et al., 2014) and higher functional connectivity between the posterior hippocampus and retrosplenial complex (Sulpizio et al., 2016) as compared with *poor* navigators.

Several factors have been proposed to affect individual differences (Wolbers and Hegarty, 2010). First of all, direct correlation among some spatial abilities, such as mental rotation, left-right discrimination (Moffat et al., 1998) and perspective-taking (Kozhevnikov and Hegarty, 2001; Hegarty and Waller, 2004), and navigational skills have been repeatedly shown. Second, levels of navigational ability seem be depending on the strategy individuals adopt for orienteering. Indeed some individuals prefer to orientate themselves by using a landmark strategy, some prefer to use a route strategy and some others a survey strategy (Pazzaglia et al., 2000) and the type of strategy/representation used affect the proficiency in navigational tasks, with the worst performances in individuals adopting the landmark strategy and best performances in those adopting the survey strategy. The preference for one type of strategies has been hypothesized to be affected by Gender. Indeed, females are more prone to adopt a landmark strategy and males a survey one (Nori et al., 2009); males are more proficient than females in survey tasks (Coluccia and Louse, 2004), with females performing worse in locating environmental features on a map of a familiar environment (the University campus; Mcguiness and Sparks, 1983). Interestingly, performances are leveled-off when females are provided with the survey map of the path they are required to learn (Montello et al., 1999), or when they may study a map or explore an environment without time limits (Piccardi et al., 2011a,c). Generally males outperformed females in acquiring new spatial knowledge from direct exposure (Montello et al., 1999), but when participants are matched for preferred strategy (i.e., Route or Survey strategy) no gender effect is observed in navigational skills (Nori et al., 2006; Nori and Piccardi, 2011, 2015; Piccardi et al., 2016).

Field Dependent/Field Independent Cognitive Style (hereafter called CS) has been recently found to affect spatial ability underlining navigational skills and individuals' predispositions toward different navigational styles (Boccia et al., 2016b, 2017a). CS has been proposed as the information processing style that characterizes the way an individual analyzes and organizes the world (Witkin, 1977). Specifically, Field-independent individuals (hereafter FI) rely on an internal frame of reference in processing and organizing environmental information and are not susceptible to deceptive environmental cues. Otherwise, field-dependent individuals (hereafter FD) rely on an external frame of reference and are susceptible to deceptive cues when identifying known elements in unknown settings. CS is usually assessed by using tasks requiring participants to detect embedded simple pictures in complex configurations, such as the Embedded Figures Test (EFT; Witkin et al., 1971), or the Rod and Frame Illusion, requiring participants to align to the vertical midline a rod in the presence of a tilted surrounding frame (Witkin et al., 1954). Both of these tasks are usually performed better by FI. CS affects a wide range of cognitive skills and tasks, especially those requiring to go beyond the information given by the setting, that is tasks requiring cognitive restructuring (Witkin, 1977). Examples of cognitive restructuring

are *disembedding* and *perspectivism*. The former refers to the ability to extract salient information from the surrounding field, whereas the latter refers to the ability to recognize and to adopt the perspective of another person (Witkin, 1977). Both these processing may be considered fundamental in the processing of topographical cues. Indeed, navigating within the environmental space requires processing and "restructuring" a huge amount of information in order to create and organize a stable mental representation of the environment. Recently, CS has been found to predict performances on spatial skills underpinning navigation, such as mental rotation and egocentric perspective taking (Boccia et al., 2016b): FI performed better than FD on both tasks. Furthermore, CS predicted individual's preferred navigational strategy, with FI more prone to prefer survey strategy than FD (Boccia et al., 2017a), regardless the gender.

Here we aimed to assess whether CS affected the acquisition of new topographical knowledge and its retrieval. With this aim, participants underwent tasks aimed at assessing the acquisition and the organization of new spatial knowledge from the direct exposure to the environment, that is (1) to learn and retrieve a path within a real environment, (2) to recognize landmarks they faced along the path and (3) to order them, (4) to trace the path on a map; the retrieval tasks (i.e., path retrieving, landmark recognition and ordering, and map drawing) were performed both immediately and after 24 h. They were also assessed for the CS by using the Embedded Figure Test (EFT). We hypothesized that CS predicted the acquisition of new spatial knowledge, especially when it has been requested to reorganize the acquired information into the map (i.e., map drawing task). Corollary, we also assessed a possible Gender-by-CS interaction. We expected that males outperformed females only among FD and that, with equal level of field dependence, gender differences would disappear.

MATERIALS AND METHODS

Participants

Fifty-Four healthy college students (mean age 24.70 ± 2.07; 28 females, $t_{(52)} = 0.04$; $p = 0.97$) took part in this study. None of the participants had a history of neurological or psychiatric disease, which was confirmed during an informal interview carried out before the test phase. All participants have normal or corrected-to-normal (soft contact lenses or glasses) vision. Moreover, all participants performed the Familiarity and Spatial Cognitive Style Scale (FSCS) (Piccardi et al., 2011b) which includes 22 self-referential statements about various aspects of environmental spatial cognition. The FSCS was used to exclude participants with self-declared topographical orientation disorders. None of the participants showed the presence of navigational deficits or developmental topographical disorientation (Iaria et al., 2005, 2009; Bianchini et al., 2010).

All participants signed a consent form before the study began. This study was approved by the local ethics committee of I.R.C.C.S. Santa Lucia Foundation, in agreement with the Declaration of Helsinki.

Assessing Field Independence: The Embedded Figure Test

As briefly reported above, the individual's predisposition toward the FD or the FI (i.e., Cognitive Style) has been classically assessed by tasks requiring participants to detect embedded simple figures in complex configurations, such as the (EFT; Witkin et al., 1971) and the hidden figures test (Ekstrom et al., 1976). Generally, FI individuals, by ignoring contextual information, are more able at detecting the embedded figures than FD individuals, who are more affected by the contextual (almost deceptive) information of the complex configurations and are less able at detecting the embedded figures in the whole configurations (Witkin, 1977; Witkin et al., 1977; Walter and Dassonville, 2011).

We used the EFT (Witkin et al., 1971) to assess individual's predisposition toward FI/FD. Participants were tested individually in a quiet, well-lit room. The participants were explained that they had to find a simple geometric shape within a larger complex figure. The experimenter presented the larger complex colored figures one-by-one for 15 s, on a 12.9 × 7.7 cm card. During the presentation time, participants were asked to orally describe the figure. After this period, the experimenter removed the complex figure and presented the simple black/white figure, for 10 s, on a card of the same size of the complex figure. Then, he/she removed the simple figure and presented once again the complex figure, asking participants to find that simple figure within the complex one. Participants were also required to advise the experimenter as soon as they found the embedded simple figure and then to trace it by using a stylus. The experimenter clocked the time. When participants said that he/she has find the simple figure, the experimenter annotated the time passed (timing): if the response was correct, that time represented the response time; otherwise, if the response was wrong, the experimenter continued to clock the time until participant produced the correct response or until 180 s had been passed. The total time was computed by summing the response time on each item. The total time was then divided by the number of items (12) to compute the overall time averaged across items. Averaged times (EFT scores) were used as the measure of the individual's Cognitive Style (CS), with lower times indicating individuals with higher predisposition toward the FI.

Navigational Abilities in Experimental Environment

Spatial Perspective Taking

The PTSOT is a spatial orientation task, proposed by Kozhevnikov and Hegarty (2001). It is a paper-and-pencil test frequently used to assess egocentric perspective taking (Hegarty and Waller, 2004). The PTSOT is composed of 12 trials. In each trial, an array of 7 objects is drawn on the top half of a 210 × 297 mm sheet. On each trial, participants are asked to imagine being placed at the position of one object in the array (imagined position), facing another object (heading direction) and to indicate the direction to a third object (the target object). A circle is depicted on the bottom half of the page. The imagined position is drawn in the center of the circle, while

the heading direction is drawn as an arrow pointing vertically up. Participants are asked to draw an arrow from the center of the circle (imagined position) to indicate the direction to the target object (Kozhevnikov and Hegarty, 2001). For each participant and trial, the absolute deviation in degrees between the individual's response and the correct direction to the target was computed (Kozhevnikov and Hegarty, 2001; Hegarty and Waller, 2004). For each participant, the total score is the average deviation across all trials (Kozhevnikov and Hegarty, 2001; Hegarty and Waller, 2004). Lower scores (i.e., lower deviation from the correct direction) corresponded to better performances.

Self-reported Assessment of Spatial Orientation Ability

The Santa Barbara Sense of Direction Scale (SBSOD) is a self-report questionnaire (Hegarty et al., 2002) that has been shown to strongly correlate with actual navigation ability (Janzen et al., 2008; Wegman et al., 2014). Following Hegarty et al. (2002), after reverse scoring, the sum of the scores for all of the items was calculated and then divided by the number of items (i.e., 15) to compute the average score across items for each participant. The SBSOD scores ranged between 1 and 7, with higher scores corresponding to a better-perceived sense of direction.

Navigational Abilities in Ecological Environment

Participants were requested to learn an out-door path within "Umberto I" general hospital campus in Rome (**Figure 1A**). The path encompassed 20 turning points, balanced across left ($N = 5$), right ($N = 7$), and straight ($N = 8$). Eight landmarks (and 8 distracters) have been selected for the landmark recognition task (see the description below).

Route Learning

The experimenter showed the path (**Figure 1A**) and asked the participant to pay attention to each landmark and turn across the path. At the end of the path (immediate-recall), the experimenter drove the participant at the starting point and asked him/her to *retrieve the path* (route learning, RL). The experimenter corrected the participants if they took the wrong decision on the turning points and, once they completed the whole path, he/she asked the participants to retrieve the path again (from the starting point) until they correctly performed the whole path. Participants who learnt the path on the first attempt were not required to retrieve the path again. All participants correctly retrieve the whole path until the second attempt. The learning score was calculated by attributing one point for each turn correctly performed, until the criterion was reached (i.e., all turns correctly performed); then it was added to the score corresponding to correct performance of the remaining attempts (up to the 2nd; maximum score: 40). For example, if the participant correctly retrieved the path by the first attempt, he/she obtained 20 on the first attempt and 20 on the second attempt, thus his/her score on RL was 40. Otherwise, if the participants correctly retrieved 18 out of the 20 turns of the path on the first attempt and all the turns on the second one, he/she obtained 18 on the first attempt and 20 to the second one. Thus, his/her score on RL was 38. Time needed to reproduce the path (seconds) was also registered and used for further analyses.

Landmark Recognition and Ordering

After the RL, the participants were presented with eight pictures of landmarks encountered along the way (e.g., a building) interspersed with eight pictures of distracters (e.g., a building similar to the actual landmark; **Figure 1B**). The participants had to indicate for each picture whether it represented the landmark encountered along the path or not (landmark recognition, LR; maximum score: 16). Then, they were asked to order the landmarks they identified (landmark ordering, LO; maximum score: 8).

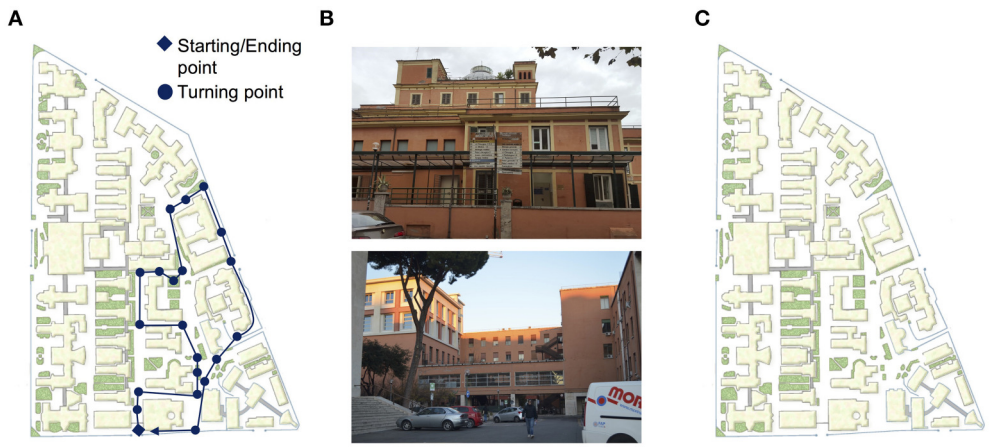

FIGURE 1 | Tasks assessing navigational abilities in ecological environment. **(A)** Path within "Umberto I" general hospital. Circles and rhombus indicate the turning points and the starting/ending point, respectively. **(B)** Example of landmark (on the top of the panel) and distracter (on the bottom of the panel) used in landmark recognition task. **(C)** Sketch map used during map drawing task.

Map Drawing

The participants were asked to draw the path on the sketch map of the "Umberto I" general hospital (map drawing, MD; **Figure 1C**). The score was calculated by attributing one point for each turn that had been correctly drawn by the participants (maximum score: 20).

Delayed Recall

One day after (about 24 h later, 24 h-retrieval), participants were requested (1) to reproduce the path they learned without additional demonstration (delayed route recall, dRR), (2) to recognize and (3) to order the landmarks (respectively, delayed landmark recognition, dLR, and delayed landmark ordering, dLO) and (4) to draw the path on the sketch map of the general hospital (delayed map drawing, dMD).

Statistical Analyses

Statistical analyses were performed by using SPSS. First, we computed Pearson's correlation coefficients among different tasks and EFT. Level of significance was set at $p = 0.05$. Thus, we performed linear regression analyses with EFT scores as predictor and score on navigational tasks (which result to be significantly correlated with EFT) as dependent variables. Significant p has been estimated by applying Bonferroni's correction for multiple comparisons.

Among these tasks, we further assessed whether gender interacted with CS in determining individuals' performances. To this aim, we calculated quartiles on EFT scores. Thus, we classified participants of the first quartile (i.e., fastest individuals) as Field Independent (FI; $N = 13$) and those of the fourth quartile (i.e., slowest individuals) as Field Dependent (FD; $N = 13$). Individuals who fell within the second and third quartiles were excluded from further analysis. Males and females were equally distributed across FI (6 females and 7 males) and FD (5 females and 8 males) individuals (Chi-squared = 0.158; $p = 0.691$). Thus, we performed a Multivariate Analysis of Variance (MANOVA), by entering Gender and CS as independent variables and performances on PTSOT, time of RL, MD, dLR, and dMD as dependent variables. Level of significance was set at $p = 0.05$ and Bonferroni's correction for multiple comparisons has been applied on pairwise comparisons.

RESULTS

The Pearson's correlation among different tasks and EFT are reported in **Table 1**. To assess whether CS predicted

TABLE 1 | Pearson's correlations.

		EFT scores	PTSOT	SBSOD	RL	Time for RL	LR	LO	MD	dRR	dLR	dLO	dMD
EFT scores	r	1	*0.436*	0.031	−0.162	*0.284*	−0.141	0.011	*−0.375*	0.098	*−0.342*	−0.158	*−0.414*
	p		*0.001*	0.824	0.242	*0.038*	0.308	0.939	*0.005*	0.479	*0.011*	0.255	*0.002*
PTSOT	r		1	−0.044	0.034	−0.010	0.008	−0.133	*−0.346*	−0.004	−0.090	−0.112	*−0.392*
	p			0.754	0.807	0.940	0.957	0.338	*0.010*	0.976	0.517	0.419	*0.003*
SBSOD	r			1	*0.351*	*−0.325*	−0.043	0.096	0.088	−0.018	−0.079	0.149	0.210
	p				*0.009*	*0.017*	0.760	0.491	0.526	0.896	0.572	0.281	0.128
RL	r				1	*−0.872*	0.180	0.108	0.151	0.006	−0.001	0.118	0.165
	p					*0.000*	0.194	0.437	0.277	0.968	0.995	0.397	0.233
Time for RL	r					1	−0.088	−0.129	−0.117	0.235	−0.006	−0.168	−0.110
	p						0.526	0.353	0.399	0.088	0.968	0.223	0.430
LR	r						1	*0.361*	0.155	0.060	*0.662*	0.237	0.104
	p							*0.007*	0.264	0.667	*0.000*	0.085	0.453
LO	r							1	0.247	−0.095	*0.366*	*0.472*	0.102
	p								0.071	0.494	*0.007*	*0.000*	0.463
MD	r								1	0.195	0.227	0.126	*0.748*
	p									0.158	0.098	0.364	*0.000*
dRR	r									1	−0.114	−0.266	0.138
	p										0.414	0.052	0.321
dLR	r										1	*0.571*	0.219
	p											*0.000*	0.111
dLO	r											1	0.183
	p												0.186
dMD	r												1
	p												

r, Pearson's correlation coefficient; p, p-value; PTSOT, Perspective Taking/Spatial Orientation Test; SBSOD, Santa Barbara Sense of Direction Scale; RL, Route Learning; LR, Landmark Recognition; LO, Landmark Ordering; MD, Map Drawing; dRR, Delayed Route Recall; dLR, Delayed Landmark recognition; dLO, Delayed Landmark Ordering; dMD, Delayed Map Drawing. Italic values indicate the r and p statistically significant.

performances on navigational tasks we performed linear regression analyses with EFT scores as predictor and score on navigational tasks (which have been found to be correlated with EFT) as dependent variable. We found that time of RL ($\beta = 0.284$; $t = 2.135$; $p = 0.038$), MD ($\beta = -0.375$; $t = -2.916$; $p = 0.005$), dLR ($\beta = -0.342$; $t = -2.625$; $p = 0.011$) and dMD ($\beta = -0.414$; $t = -3.283$; $p = 0.002$), as well as performances on PTSOT ($\beta = 0.436$; $t = 3.494$; $p = 0.001$), were significantly predicted by EFT scores. Effects on MD, dMD, and PTSOT were still significant when Bonferroni's correction for multiple comparisons was applied (significance level $p = 0.01$).

Concerning the results of MANOVA, we found a main effect of Gender on MD [$F_{(1, 22)} = 6.432$; $p < 0.05$; Partial Eta Squared = 0.226; Observed Power = 0.679] and dMD [$F_{(1, 22)} = 14.699$; $p < 0.01$; Partial Eta Squared = 0.401; Observed Power = 0.956]. In both cases, males (*MD*: $M = 14.530$, $SD = 6.334$; *dMD*: $M = 16.800$, $SD = 5.226$) outperformed females (*MD*: $M = 8.450$, $SD = 7.647$; *dMD*: $M = 9.00$, $SD = 6.943$). We also found a main effect of CS on PTSOT [$F_{(1, 22)} = 9.549$; $p < 0.01$; Partial Eta Squared = 0.303; Observed Power = 0.840; **Figure 2A**] and MD [$F_{(1, 22)} = 4.549$; $p < 0.05$; Partial Eta Squared = 0.171; Observed Power = 0.532; **Figure 2B**]. In both cases, FI performed better than FD. Actually, FI individuals showed lower deviation from correct response on PTSOT ($M = 34.282$, $SD = 19.477$) than FD individuals ($M = 64.526$, $SD = 33.393$). FI also performed better ($M = 14.230$, $SD = 7.178$) than FD ($M = 9.690$, $SD = 7.239$) on MD. Interestingly, we found that Gender and CS interacted on Time for RL [$F_{(1, 22)} = 8.057$; $p < 0.05$; Partial Eta Squared = 0.268; Observed Power = 0.774] and dMD [$F_{(1, 22)} = 5.325$; $p < 0.05$; Partial Eta Squared = 0.195; Observed Power = 0.597]. Concerning Time for RL, females were slower than males only in the FD group of participants ($p = 0.040$, Bonferroni's correction for multiple comparisons; **Figure 2C**). Females performed worse than males only in FD group ($p <$

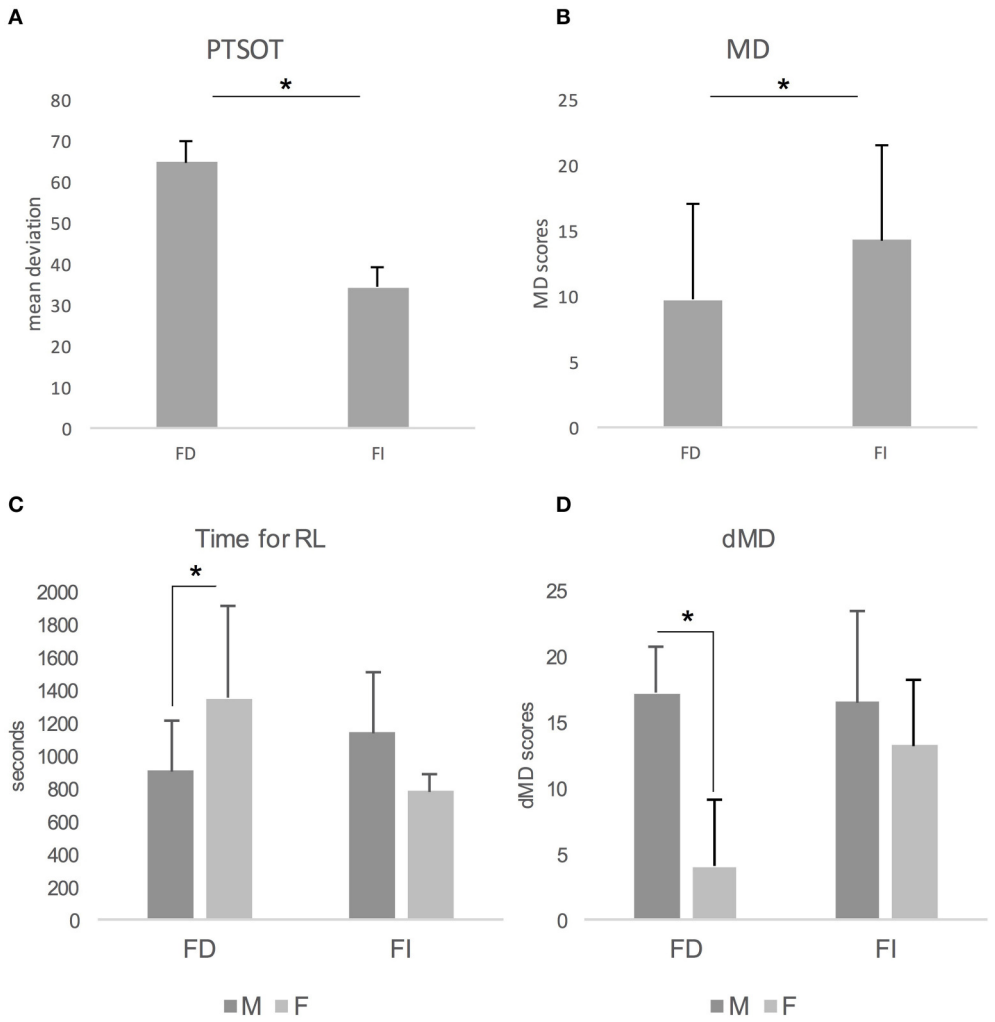

FIGURE 2 | Effect of Cognitive Style on PTSOT **(A)** and Map Drawing task **(B)**. Gender by Cognitive Style interaction on time needed to learn the path **(C)** and on delayed map drawing **(D)**. Bars depict mean and standard deviation of performances. FI, Field Independent; FD, Field Dependent; M, Males; F, Females; PTSOT, Perspective Taking/Spatial Orientation Test; RL, route learning; MD, map drawing; dMD, delayed map drawing.

0.001, Bonferroni's correction for multiple comparisons) also on dMD (**Figure 2D**).

DISCUSSION

Here we found that Field dependence/independence Cognitive Style (CS) affects performance on several navigational tasks, that is map drawing task (both immediate and delayed recall) and PTSOT. FI individuals performed better than FD ones in map drawing task and PTSOT. Corollary, we found that gender interacted with CS in time for route learning and delayed map drawing task, and gender differences were detectable just in FD individuals.

CS is a pervasive characteristic of individuals' perceptual and intellectual functioning which cannot be affected by experience or learning (Witkin, 1967, 1977). As reported in the introduction, CS affects a wide range of abilities, such as mental rotation and egocentric perspective taking, especially when, as often happens in navigation, cognitive restructuring is required (Boccia et al., 2016b). Indeed, to successfully orientate within the environmental space, environmental inputs and knowledge need to be continuously restructured; individuals have to continuously restructure information given by the context (i.e., the current vista space and the environmental space), to online update both spatial cues in the environment and their current relative (one in respect of the other) and absolute (in respect of allocentric references) positions, to process spatial computations of Euclidean/metric environmental features (for example, distances), to translate the egocentric representations of the environment into allocentric ones (and vice versa), to monitor the route progression and to plan novel routes, to develop the online representations of the environment and to retrieve the offline (i.e., previously acquired) topographical knowledge (Wolbers and Hegarty, 2010).

Here we assessed the effects of CS on spatial navigation within a real environmental space, by testing the acquisition (*route learning task*) and retrieval (*delayed route recall*) of a novel open-field environment; also, landmark recognition and the knowledge of their relative spatial positions (*landmark recognition* and *landmark ordering*) were evaluated. Cognitive restructuring was fostered by asking participants to transform route learning into a 2D map-like representation (*map drawing*).

In agreement with our hypothesis, FI individuals were more able than FD in the map drawing task and in reporting learned path into a map (both immediately and after 24 h). Even if no significant difference was observed, it should be noticed that regression showed a trend in FI participants toward less time required for learning the path (i.e., total time required to learn the path) and better ability to recognize landmarks after 24 h. Indeed, all participants, independently from their CS, were able to learn the path within the second attempt, to recognize all the landmarks and to correctly retrieve the order in which they had met them along the route. Instead, CS strongly affects the map-drawing skill, which is significantly predicted by performances on EFT. These results suggest that environmental navigation skills are not generally affected by CS, which plays a very specific role on abilities, such as those involved in map drawing, related to the "creation" of a flexible mental representation of the environmental space.

This interpretation is supported by a previous study showing that FI individuals are more likely to prefer and adopt a survey strategy, while FD ones are very likely to prefer and adopt a landmark strategy (Boccia et al., 2017a). These results allow to hypothesize that the evolved navigational strategy, namely the survey strategy, may be easily accessible to individuals with a FI CS, but not to those with a FD CS. As a consequence, FI individuals should show better performances whenever the vista representation alone and route/landmark strategies are not sufficient for achieving a navigational goal or whenever a survey/allocentric representation of the environmental space is required. Some results support this hypothesis. Indeed, Boccia et al. (2017a) found FI individuals were more proficient in performing the "survey" tasks, that is tasks requiring participants to use an abstract, internal, object-based representation of the space, which almost corresponds to a "bird's-eye viewpoint" (i.e., survey representation) (Nori and Giusberti, 2006) and in present study FI were significantly better in both the map drawing tasks. The ability to represent in a map what has been acquired while exploring a novel environment depends on the ability to develop a cognitive map, that is an allocentric, survey representation of the environment (Iaria et al., 2007). Following some current navigational models, allocentric representations are developed by familiarization with the environment (Siegel and White, 1975; Montello, 1998) and higher is the familiarity better is the allocentric representation. In present study, however, the better performance on map drawing in FI individuals cannot be explained as an effect of familiarity, since their time of familiarization with the novel environment they had never been exposed before did not differed from that of FD. Thus, differences in performance should depend on differences in the way the navigational features are processed depending on individuals' CS. FI individuals are able to process navigational information in an evolved allocentric representation in a quicker and more effective way than FD; the effect of CS on the ability to develop, store and retrieve allocentric representations is stable across time, since, CS predicted individual's performance in map drawing not only soon after the end of the learning (immediate-retrieval), but also in the retest after 24 h (24 h- retrieval). Present study does not allow to understand if an increased familiarization would improve FD's performances, since in our paradigm a fixed learning experience was provided and future studies are necessary for understanding if individuals with very high levels of FD are able to develop an evolved allocentric representation if provided of extensive familiarization. However, present results clearly underline the advantage of FI in developing map representation even in absence of differences from FD in learning novel routes in a previously unknown place.

Why FI individuals are more proficient in developing the survey/allocentric representations? To perform the map drawing tasks, individuals had to transform the environmental information coded during an egocentric experience of the "vista space" (Wolbers and Wiener, 2014) into an allocentric, map-like representation of the "environmental space" (Wolbers

and Hegarty, 2010; Wolbers and Wiener, 2014), in which the positions of the landmarks, as well as the relative distances between landmarks, have to be represented regardless of the individual's position. Thus, this task fostered the individual's ability to "restructure" the egocentrically acquired route knowledge into an abstract, survey representation of the environmental space. As stated above, FI individuals usually rely on an internal frame of reference in restructuring the environmental information. Their higher ability in map drawing task may be *generally* due to the fact that they rely on the internal frame of reference to proficiently restructure the "route" knowledge about a given environment into the corresponding "survey" representation. In this light, the survey representation of the environmental space should rise from an internally driven reorganization of the egocentrically acquired spatial knowledge of neighboring vista spaces. Another characteristic of FI, that is *perspectivism*, should also play a role in the better performance of FI individuals. As reported above, field-independent individuals are usually more able than field-dependent individuals in adopting the perspective of another person. This capability to "look from another point of view" could ease the spatial computation allowing the translation of egocentrically acquired route knowledge into map-like, survey representation. This possible role of *perspectivism* in developing survey representations is consistent with finding that map drawing is significantly correlated both with CS and performances on egocentric perspective taking task (see **Table 1**).

As reported above, CS only marginally affected the time for learning and the delayed landmark recognition tasks without no significant group differences between FI and FD individuals. Also, we did not find any effect of the CS on Route Learning and Landmark Recognition (immediate-recall). Both these tasks do not require cognitive restructuring, since they tap on the memory for the path and landmark in the vista space, as it happens for Map-drawing task, but also they do not involve perspectivism, since no change of the point of view is required for deciding if a landmark was on the route and if it follows or not a another given landmark.

Taken together, our data suggest that FI/FD CS have specific effects only on some of the navigational skills, and in particular only on the ability to build up complex representations of the environmental space starting from the knowledge acquired form the vista space, perhaps affecting also the ability to translate the format of the environmental knowledge from one type of frame of reference to another (for example from the egocentric to the allocentric frame of reference and vice versa). Following this interpretation, the effects of CS should be evident in all the tasks requiring a reorganization of environmental knowledge, even those in which no direct spatial translation is required, such as, for example, when a detour from a familiar route is required to cope with a blocked-route. In this case the task forces individuals to "restructure" previous knowledge to solve the navigational "request" (Hirshhorn et al., 2012) moving form a vista-space representation to an environment-space one.

Present results confirm those about egocentric perspective taking task obtained by Boccia et al. (2016b), who showed that CS predicts performances on PTSOT. In this previous study, CS was assessed by using the Group Embedded Figure Test (GEFT), a test differing from the EFT used in present study in different aspects. Indeed, despite using the same stimuli, the two tests differ both in the administration (the GEFT is administered to groups of participants simultaneously, whereas the EFT is administered individually) and in behavioral indexes used for scoring, since the GEFT uses the accuracy as the index of CS, while the EFT uses the response time. Results of present study, thus, not only confirm the previous one, but also offer a convergent evidence about the relation between the egocentric perspective taking and the CS.

As a corollary aim, we assessed whether CS interacted with Gender in determining navigational skills. As a group, females performed worse than males on map-drawing, both immediately and after 24 h. These results are consistent with previous literature about gender differences in navigational skills (Coluccia and Louse, 2004). However, we also found that gender interacts with CS on the time needed to learn the path (i.e., time for route learning) and delayed map-drawing (interaction has been not detected for immediate map-drawing). Females performed worse than males only if they are FD individuals, while no gender difference was present in the group of FI individuals. This result mirrors that of a previous study finding that when men and women were matched for their spatial style they do not show differences in their spatial orientation ability (Nori and Giusberti, 2003, 2006). Also, the present result points toward a pivotal role of CS in gender-related differences in environmental navigation, prompting to put attention to this dimension in future investigations about gender differences. Indeed, the observation that only field dependent females show worst performances than males suggests the possibility that contrasting results about gender differences in navigational and topographical skills can be due to the presence of different percentage of FI and FD women in different studies, so that studies in which a higher percentage of FI is included in the female group failed in finding gender differences that were instead present in the studies in which the female group included a higher percentage of FD.

Interestingly, even if CS predicted performance on several navigational tasks (see above for detailed discussion about the effect of CS on map-drawing task and egocentric perspective taking), there is no significant effect for self-reported navigational skills. This is consistent with a previous investigations (Boccia et al., 2016b), confirming that individuals' meta-cognition about their own spatial abilities do not always correspond to their actual capability. Also, here we found that self-reported navigational skills on SBSOD were significantly correlated with performances and time of route learning, but they were not correlated with performances on the map-drawing task, which is the "navigational skills" mainly affected by CS.

The present findings may have some importance in the field of developmental and acquired topographical disorientation. Topographical disorientation has been described as a consequence of acquired brain damage (Aguirre and Esposito, 1999), congenital malformation (Iaria et al., 2005), normal and pathological aging (Boccia et al., 2016c; Nemmi et al., 2017) as well as in individuals who never develop such an ability (Developmental Topographical Disorientation; DTE) (Iaria

et al., 2009, 2014; Bianchini et al., 2010, 2014; Iaria and Barton, 2010; Iaria, 2013; Palermo et al., 2014a,b; Nemmi et al., 2015). Further studies should investigate whether and how CS affects development of topographical orientation ability and/or loss of such an ability due to acquired brain damage or cognitive decline in pathological and normal aging.

The neural mechanisms underlying the relation between CS and environmental navigation have never been explored. Neuroimaging investigations about the neural underpinnings of field-independence/field-dependence suggest a pivotal role of the superior parietal lobe (Walter and Dassonville, 2011; Lester and Dassonville, 2014), an area that has been repeatedly found to be engaged in spatial navigation (Boccia et al., 2014). Whether and how the parietal lobe is involved in the relationship between CS and spatial navigation is a fascinating issue that further studies need to address. However, some data in literature support this hypothesis. Following the model proposed by Byrne and colleagues (Byrne et al., 2007), in the parietal lobe there should be the neural substrate of the "egocentric parietal window" that allows the egocentrically coded information in the parietal lobe to access the allocentrically stored information in the medial temporal lobe, in service of the mental imagery and the spatial navigation. Neuroimaging evidence in both humans (Boccia et al., 2016d, 2017b) and primates (Kravitz et al., 2011) seems to support the model.

In conclusion, the present results confirm that CS affects environmental navigation, especially when a map-like representation is required. Thus, FI is pivotal to restructure the environmental information in a global and flexible long-term representation of the environment, namely the cognitive map, as well as in easing the changes of perspective which allow individuals to re-orient and recognize places from a point of view different from the familiar one.

ETHICS STATEMENT

This study was carried out in agreement with the Declaration of Helsinki with written informed consent from all subjects. The protocol was approved by the local ethical committee of Santa Lucia Foundation.

AUTHOR CONTRIBUTIONS

All the authors conceived the study. MB and FV prepared the stimuli and the tasks. FV collected the data and MB analyzed the data. MB wrote a first draft of the manuscript. All the authors contributed to the revision of this first draft and the discussion of the results.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 23 August 2017
doi: 10.3389/fphar.2017.00550

Differences in Spatial Memory Recognition Due to Cognitive Style

*Laura Tascón[1], Maddalena Boccia[2,3], Laura Piccardi[3,4] and José M. Cimadevilla[1]**

[1] Department of Psychology, University of Almeria, Almeria, Spain, [2] Department of Psychology, 'Sapienza' University of Rome, Rome, Italy, [3] Cognitive and Motor Rehabilitation Unit, IRCCS Fondazione Santa Lucia, Rome, Italy, [4] Department of Life, Health and Environmental Sciences, L'Aquila University, L'Aquila, Italy

Field independence refers to the ability to perceive details from the surrounding context as a whole and to represent the environment by relying on an internal reference frame. Conversely, field dependence individuals tend to focus their attention on single environmental features analysing them individually. This cognitive style affects several visuo-spatial abilities including spatial memory. This study assesses both the effect of field independence and field dependence on performance displayed on virtual environments of different complexity. Forty young healthy individuals took part in this study. Participants performed the Embedded Figures Test for field independence or dependence assessment and a new spatial memory recognition test. The spatial memory recognition test demanded to memorize a green box location in a virtual room picture. Thereafter, during ten trials participants had to decide if a green box was located in the same position as in the sample picture. Five of the pictures were correct. The information available in the virtual room was manipulated. Hence, two different experimental conditions were tested: a virtual room containing all landmarks and a virtual room with only two cues. Accuracy and reaction time were registered. Analyses demonstrated that higher field independent individuals were related to better spatial memory performance in two landmarks condition and were faster in all landmark condition. In addition, men and women did not differ in their performance. These results suggested that cognitive style affects spatial memory performance and this phenomenon is modulated by environment complexity. This does not affect accuracy but time spent. Moreover, field dependent individuals are unable to organize the navigational field by relying on internal reference frames when few landmarks are available, and this causes them to commit more errors.

Keywords: spatial memory, field dependence/independence, virtual reality, embedded figures test, environmental complexity

OPEN ACCESS

Edited by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico

Reviewed by:
David Giofrè, Liverpool John Moores University, United Kingdom
Andrea Bosco, Università degli Studi di Bari Aldo Moro, Italy

***Correspondence:**
José M. Cimadevilla
jcimadev@ual.es

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *28 June 2017*
Accepted: *07 August 2017*
Published: *23 August 2017*

Citation:
Tascón L, Boccia M, Piccardi L and Cimadevilla JM (2017) Differences in Spatial Memory Recognition Due to Cognitive Style. Front. Pharmacol. 8:550. doi: 10.3389/fphar.2017.00550

INTRODUCTION

Spatial memory is a cognitive ability that permits the recollection of information about the space, its layout and locations (Castree et al., 2013). Spatial information can be analysed differently and allows diverse possibilities of action in a given spatial task (Kyritsis et al., 2009; Li et al., 2016). The distinct preferences to perceive and organize the information about the surrounding space are known as cognitive style (CS) (Hayes and Allinson, 1998; Smith and Riding, 2004; Kyritsis et al., 2009). Two opposite CS can be found: on the one hand, field independent (FI) participants

can manage a holistic environmental representation, while, at the same time, they can perceive parts as a whole. On the other hand, field dependent (FD) subjects focus their attention on single environmental features by analyzing them individually (Witkin, 1977; Kyritsis et al., 2009).

The Embedded Figures Test is a paper-and-pencil task used to define the CS (Witkin et al., 1971). Participants are requested to search for a simple figure hidden in a complex one. Usually, FD individuals take longer to perform the task.

It is worthy to note that CS can predict performance on different spatial tasks. For instance, FI people are good at object rotation, perspective taking and using no-rotating maps (Boccia et al., 2016; Li et al., 2016). They have also been reported to handle more complex and flexible environmental representations (i.e., map-like representation) as compared with FD (Boccia et al., 2017).

Moreover, gender differences have been found in spatial tasks (Coluccia and Louse, 2004; Iachini et al., 2005; Cimadevilla et al., 2011; Piccardi et al., 2011a,b; Nori et al., 2015; León et al., 2016; Palmiero et al., 2016; Tascón et al., 2016, 2017). It is important to highlight that dimorphism depends on the task difficulty level, disappearing with low and very high demands (Coluccia and Louse, 2004; Nori and Piccardi, 2011; León et al., 2014; Tascón et al., 2017).

In addition to this, men and women are prone to use different strategies and spatial information to solve the same tasks (Coluccia and Louse, 2004; Driscoll et al., 2005; Woolley et al., 2010; Nori et al., 2015). In accordance with the Siegel and White's Model (see Siegel and White, 1975), the spatial CS corresponds to the type of information individuals select to navigate and orientate themselves in the environment. Generally speaking, women normally adopt a "landmark style" so as to "beacon" towards a salient landmark, using a sort of figurative memory, or a "route style" to navigate relying on the memory of the paths that connect different landmarks. Both styles are related to the use of egocentric strategies. Unlike them, men prefer to use "survey style," a global map-like environmental representation associated with the use of allocentric strategies (Pazzaglia and De Beni, 2001; Coluccia and Louse, 2004; Nori et al., 2006). Considering field dependence/independence continuum, men have been reported to be FI while women are FD (Boccia et al., 2016).

On the other hand, spatial abilities have been assessed in humans using different methods. Virtual reality-based tasks have proved to be more accurate and useful to detect brain damages than classical neuropsychological tests (Cimadevilla et al., 2014). Indeed, spatial orientation in virtual environments is considered realistic enough to activate the same mechanisms involved during navigation in real environments both at behavioural and at neural levels (Aguirre et al., 1996).

Recently, a new spatial task was developed for assessing spatial memory in humans (Tascón et al., 2017). It demands participants to remember locations in a spatial recognition test and it has been reported as a good gender discrimination (Tascón et al., 2017).

Abbreviations: 2L, two landmarks condition; AL, all landmarks condition; CS, cognitive style; FD, field dependence/dependent; FI, field independence/independent.

The aim of the present study was to determine the effect of CS and gender on the performance in the spatial recognition task. According to previous literature, FI are better in handling spatial information, like perspective taking, a kind of process involved in the spatial recognition task used in this work. In addition, FI are more capable than FD in extracting important environmental information required for an accurate orientation. Taking into account that women are more often FD than men and two contexts with a different amount of landmarks will be used, we hypothesize that FI will show a better performance in spatial recognition than FD and we also expect that women will do better when all landmarks are available.

MATERIALS AND METHODS

Participants

The sample was made up of 40 undergraduate College students from "Sapienza" University, Rome (Italy). Twenty of them were men (Mean age = 25.7; *SD* = 2.8) and the other half women (Mean age = 25.7; *SD* = 2.3). None of them had a history of neurological or psychiatric diseases, which was later confirmed during an informal interview carried out before the test phase. In addition, all participants performed the Familiarity and Spatial Cognitive Style scale (FSCS; Piccardi et al., 2011c) which included 22 self-referential statements about various aspects of environmental spatial orientation. All participants self-classified themselves as having a "good or quite good sense-of-direction," as evaluated by filling in the FSCS. Indeed, this scale was used to ensure that participants did not suffer from topographical orientation disorders. None of the participants showed the presence of navigational deficits or developmental topographical disorientation (see Iaria et al., 2005, 2009; Bianchini et al., 2010).

For demographic details see **Table 1**.

The study was developed under the European Community Council Directive, 2001/20/EC for biomedical research in humans. All subjects gave written informed consent.

Instruments and Procedure

The individual's predisposition toward the FD or the FI (i.e., CS) is usually assessed by tasks requiring participants to detect embedded simple figures in complex configurations (e.g., Witkin et al., 1971; Ekstrom et al., 1976). In these tasks, FI individuals, by ignoring contextual information, are better at detecting the embedded figures than FD individuals, who are more affected by the contextual information of the complex configurations and are less able at detecting the embedded figures in the whole configurations (Witkin, 1977; Witkin et al., 1977; Walter and Dassonville, 2011).

TABLE 1 | Distribution of the sample in gender and age.

Males	*FI* = 11 *FD* = 9 Total = 20	*N* = 40 Mean age = 25.7 *SD* = 2.5
Females	*FI* = 9 *FD* = 11 Total = 20	

In the present study, we adopted the Embedded Figures Test (EFT) for assessing the participants' CS. The EFT is a paper-and-pencil test developed by Witkin et al. (1971) to analyse how an individual perceives and processes the surrounding field. It consists of a collection of cards 12.9 × 7.7 cm with complex and simple figures. Those simple figures are uncoloured and are formed by a single line. Complex figures are composed of a conjunction of small simple and multi-coloured figures. Each simple figure is hidden in the complex one. That is, the contour of the simple figure is formed by several substructures of the complex, so the simple one cannot be easily identified.

Each trial started showing a complex figure for 15 s. During this time participants had to describe it out in loud voice. Thereafter, the card with the simple figure overlapped the complex one for 10 s. After that, the experimenter removed the card with the simple figure and the participants had to find the contour of it inside the complex. They were instructed to inform the experimenter as soon as they found the simple figure and then to trace it by using a stylus. When a participant believed to have found the simple figure, the experimenter annotated the elapsed time (timing): if the response was correct, that time represented the response time; otherwise, if the response was wrong, the experimenter continued to clock the time until the participant reported the correct response or until 180 s had passed. The total time was computed by summing up the response time on each item, the result being divided by the number of items (Piccardi et al., 2011a) in order to compute the overall time averaged across items. Averaged times (EFT scores) were used as the measure of the individual's CS, with lower times indicating individuals with higher predisposition towards the FI.

As a scale for dividing subjects into FI and FD does not exist, and taking into account that the individual's predisposition to be field in/dependent is along a continuum, we decided to sort participants according to the median of the averaged times on the EFT. In such a way we divided participants into two groups: FI (i.e. higher times than median, that is faster individuals in detecting embedded figures) and FD (i.e. lower times than median, that is slower individuals in solving the EFT) groups.

The spatial recognition test (Tascón et al., 2017) was displayed on a Hewlett Packard 2600-MHz notebook equipped with 3 GB of RAM and a 15.4 Thin Film Transistor (TFT) colour screen (1920 × 1200 pixels). The recognition test was implemented in MATLAB using Cogent 2000 (Well- come Laboratory of Neurobiology, UCL, London, www.vislab.ucl.ac.uk/cogent.php).

The spatial recognition task was based on the Almeria Spatial Memory Recognition Test (ASMRT) (Tascón et al., 2017). Instructions along with an example were displayed on the screen. The spatial task included two phases: learning and recognition phase. In the learning phase a picture showed a square virtual room with 9 boxes (3 × 3), one of them in green color. Participants were asked to memorize the green box location. No time limits were set. The recognition phase started when the space-key was pressed. A total of ten pictures in the virtual room was shown one by one. The virtual room was shown again with 9 boxes, one of them in green color. Participants had to decide if the green box corresponded spatially to the one of the sample phase. They had to provide positive or negative responses (yes/no) by pressing two buttons on the keyboard. The viewpoint changed across the pictures (see **Figure 1**). Five pictures represented correct locations. Both accuracy and reaction time were automatically recorded.

Based on the fact that FI and FD individuals perform differently in complex and simple environments, two contextual conditions were administered. In the first one, all possible landmarks (AL) were available in the virtual room represented in the picture (see **Figures 2A,B**). Every room wall but one had one or more cues. In the two landmarks condition (2L) a door and a picture were available in adjacent walls (see **Figures 2C,D**).

Statistical Analyses

The median (33.06) of the averaged times on the Embedded Figures Test (in seconds) was used to divide the group into FI (i.e., higher times than median) and FD (i.e., lower times than median) groups. A chi-square was used to determine if the proportion of men and women changed in FD and FI groups.

The number of correct answers and time spent in every condition (AL and 2L) were analysed using a two-way ANOVA (Gender x CS). Tukey test was applied for post-hoc analyses and differences were considered statistically significant for $p < 0.05$. STATISTICA 10 was used to run analyses.

RESULTS

A chi-square test showed that proportion of men and women did not differ in FI and FD groups, $X^2 = 0.4$, $p = 0.527$.

All Landmarks Condition (AL)

Accuracy (Number of Correct Answers)

ANOVA (Gender × CS) did not reveal any significant main effect of Gender $F_{(1, 36)} = 0.001$, $p = 0.977$, $\eta_p^2 = 0$, CS $F_{(1, 36)} = 2.594$, $p = 0.116$, $\eta_p^2 = 0.06$, nor Gender × CS interaction $F_{(1, 36)} = 0.065$, $p = 0.801$, $\eta_p^2 = 0.002$.

Latency

The time to perform the recognition task was analyzed with ANOVA (Gender × CS) and revealed a significant main effect of CS $F_{(1, 36)} = 5.265$, $p = 0.02$, $\eta_p^2 = 0.13$. No differences emerged neither in Gender factor $F_{(1, 36)} = 0.022$, $p = 0.88$, $\eta_p^2 = 0.001$, nor Gender × CS interaction $F_{(1, 36)} = 0.141$, $p = 0.709$, $\eta_p^2 = 0.004$. FI group response was faster in the recognition task (1162.7 vs. 1413 ms for FI and FD groups, respectively) (see **Figure 3**).

Two Landmarks Condition (2L)

Accuracy (Number of Correct Answers)

ANOVA (Gender x CS) disclosed a significant main effect of CS $F_{(1, 36)} = 6.505$, $p = 0.015$, $\eta_p^2 = 0.15$. No significant main effect was found either in Gender factor $F_{(1, 36)} = 0.027$, $p = 0.869$, $\eta_p^2 = 0.001$ or in Gender × CS interaction $F_{(1, 36)} = 0.777$, $p = 0.383$, $\eta_p^2 = 0.02$. FI participants obtained a higher number of correct answers than those in the FD group (9.66 vs. 9.11 correct answers for FI and FD groups, respectively) (see **Figure 4**).

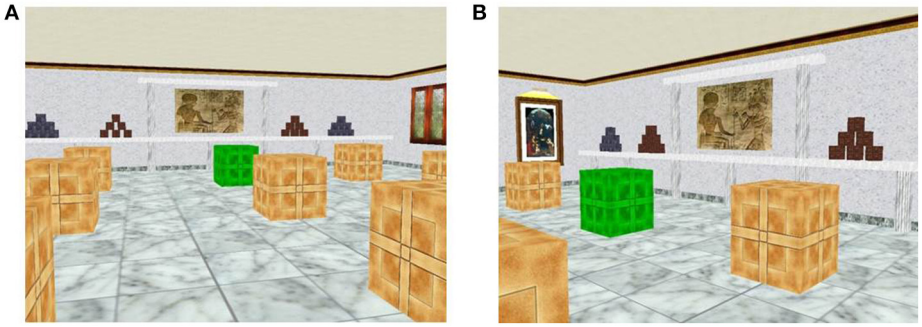

FIGURE 1 | Two pictures from the spatial recognition task. **(A)** Learning phase: participants have to memorize the location of the green box. **(B)** Recognition phase: individuals have to indicate if the green box is in the same location than in the learning phase. Note that ten pictures were used in the recognition phase.

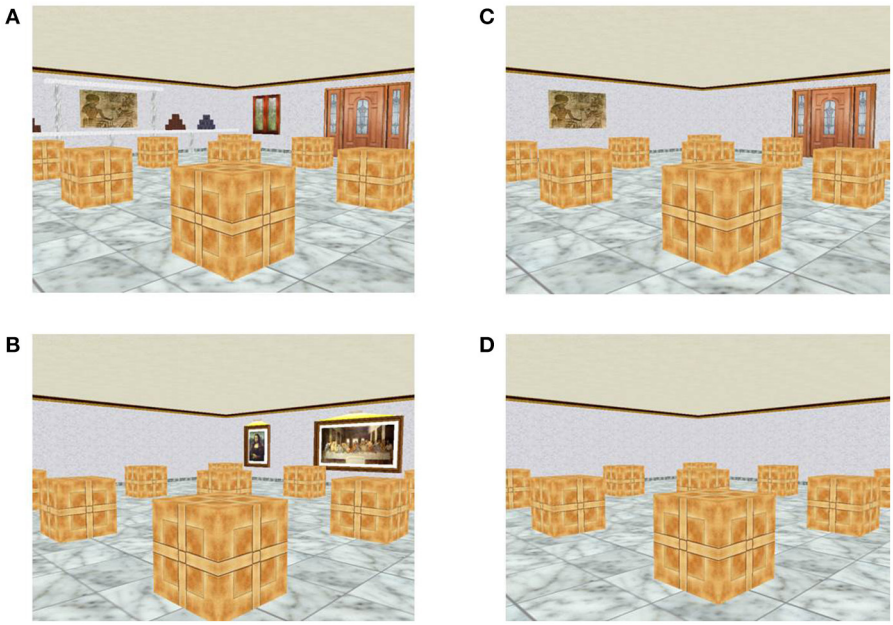

FIGURE 2 | Different conditions applied in the spatial recognition task. **(A,B)** Different perspectives of the room with all landmarks available (AL condition). **(C,D)** Different perspectives of the room with only two landmarks available (2L condition). Note that two walls did not contain any landmark.

Latency

ANOVA (Gender x CS) did not reveal any effect of Gender $F_{(1, 36)} = 0.295$, $p = 0.591$, $\eta_p^2 = 0.008$, CS $F_{(1, 36)} = 2.626$, $p = 0.114$, $\eta_p^2 = 0.06$ nor Gender × CS interaction $F_{(1, 36)} = 0.017$, $p = 0.896$, $\eta_p^2 = 0.0$.

For means and SD see **Table 2**.

DISCUSSION

Relationship between CS and spatial memory performance was assessed in this study. Analyses revealed that FI participants were more accurate than FD when few landmarks were available in the environment (2L) and they were faster than FD when all landmarks were available (AL). Note that in both conditions the virtual room was the same although the only significant change was to be found in the number of cues available.

Taking into account that FI outperform FD in tasks where spatial information needs to be cognitively handled (Witkin, 1977), such as mental object rotation, perspective taking and non-rotating maps usage (Boccia et al., 2016; Li et al., 2016), it is not unusual that they made fewer errors than FD in the 2L condition. FI individuals are more capable than FD in extracting prominent information from the environment and putting them in other's perspective to imagine what they are looking at Witkin (1977). The capacity to extract the prominent information is named disembedding and the ability to imagine other's perspective is known as perspectivism (Witkin, 1977) and both are essential for performing the spatial memory recognition task used in this study. Hence, participants needed

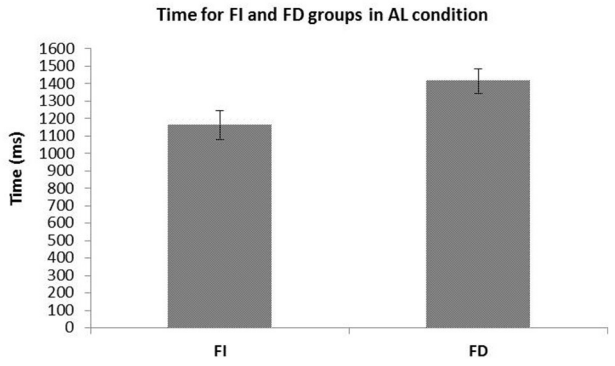

FIGURE 3 | Mean and S.E.M. of time by Field Independent (FI) and Field Dependent (FD) groups to perform the recognition task with all landmarks available. The FI group was faster than the FD one.

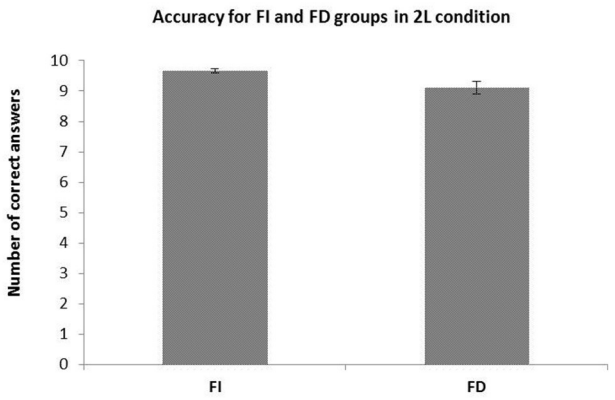

FIGURE 4 | Mean and S.E.M. of number of correct answers for Field Independent (FI) and Dependent (FD) groups in the recognition task with only two available landmarks. The FI group showed a higher accuracy.

TABLE 2 | Mean and SD for gender and cognitive style.

			Mean	SD
AL	Accuracy	Men	9.5	0.72
		Women	9.46	0.6
		FD	9.65	0.14
		FI	9.31	0.14
	Latency	Men	1,267	383
		Women	1,308	327
		FD	1,413	306
		FI	1,162	357
2L	Accuracy	Men	9.43	0.67
		Women	9.34	0.76
		FD	9.11	0.89
		FI	9.66	0.3
	Latency	Men	1,447	455
		Women	1,398	348
		FD	1,523	288
		FI	1,323	475

to remember one picture and interpret locations from other viewpoints.

In the complex environment, once more we found that FI outperformed FD participants, although group differences emerged on latencies. It is therefore likely that FD individuals could not extract important spatial information and take advantage of it. These findings are in line with subjects' spatial style, where "landmark style" participants are prone to analyse useless details of the environment making difficult their spatial orientation (Siegel and White, 1975; Piccardi et al., 2016). Piccardi et al. (2016) found that "landmark style" subjects were able to recall familiar landmarks but they did not relate them with spatial information. In this regard, they proved to be poor navigators. The eye movement pattern of "landmark style" individuals is characterised by a greater number of fixations of short duration focusing their exploration on the path and related targets. However, survey style individuals explored the environment more comprehensively, focusing their attention on salient cues (Piccardi et al., 2016). In the current research, FD participants may have analysed useless spatial information and, even in the absence of different accuracy, the time required to complete the task was higher for FD than FI. Having excessive information available could affect the FD individuals' performance, since they do not rely on important spatial information. They may spend more time because they use external reference frames which are not reliable for identifying cues in unknown settings (Witkin, 1977).

Regarding gender, Siegel and White (1975) have noted that there are different strategies to solve spatial tasks depending on the information chosen to navigate. These strategies are named "spatial styles." Some studies have found that women are usually prone to use "landmark" or "route styles," where egocentric information is necessary. On the contrary, men usually choose a "survey style," which implies allocentric information and capacity to represent the environment as a map (Coluccia and Louse, 2004; Nori et al., 2006; Nori and Piccardi, 2015). This is evident in tasks where they have to indicate how to reach a target: men normally provide cardinal points and distance information whereas women prefer to add information about landmarks (Miller and Santoni, 1986; Ward et al., 1986; Schmitz, 1997; Dabbs et al., 1998; Denis et al., 1999). This supports that FD and FI may be related to "landmark" and "survey styles," respectively (Boccia et al., 2017). "Route style" individuals could be both FD and FI since both men and women use this type of orientation (e.g., Boccia et al., 2017).

No gender differences have been revealed in our study. It is well known that dimorphism requires an optimum level of difficulty. When the level of difficulty is low both genders performed accurately. Conversely, very high difficulty levels produce an increase of errors which also increase dispersion and reduce the possibility of finding group differences (Cánovas et al., 2008; León et al., 2014; Tascón et al., 2017). In this study we have

shown that men and women displayed a similar performance. We presume that increasing difficulty would disclose a differential performance in both genders, as revealed by Tascón et al., (León et al., 2014) in similar tasks with higher difficulty levels. Another possible interpretation may be related to the distribution of men and women in the FD and FI groups, even if literature shows that women are generally more FD than men, in our sample men and women are equally distributed in the two groups. Boccia et al. (2017) demonstrated that women and men with the same CS did not differ in their performance in spatial memory tasks, adopting similar strategies. Moreover, when both gender adopted the same strategies i.e., military pilots with high spatial abilities, gender differences never appear (Verde et al., 2013, 2015, 2016).

The sample could be a limitation of this study. Although the sample was not too small according to the number of participants included in other studies using the same task (Tascón et al., 2016, 2017), when assessing cognitive styles samples are slightly bigger (Boccia et al., 2016, 2017). A bigger group would allow limiting FI and FD groups to those individuals with extreme values.

As a conclusion, it is important to highlight that different spatial contexts can modify the performance in spatial tasks depending on the CS assumed. FD participants use landmark information to navigate, so a very complex environment makes them not to focus on essential spatial information like landmarks relationship that would facilitate task resolution. FI individuals rely on internal frame references that make them better navigators.

AUTHOR CONTRIBUTIONS

LT, MB, LP, and JC: Conception and design, data collection, interpretation, critical revision, and manuscript preparation.

FUNDING

This work was supported by MINECO (PSI2015-67442-P) and by the Ministry of Education, Culture and Sport of Spain [FPU2013/02655].

ACKNOWLEDGMENTS

We thank Jose R. Ibañez for his help with English. The research was conducted in the absence of any commercial or financial relationships.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 26 September 2017
doi: 10.3389/fphar.2017.00668

Negative Facial Expressions – But Not Visual Scenes – Enhance Human Working Memory in Younger and Older Participants

*Flávia Schechtman Belham[1,2], Maria Clotilde H. Tavares[1], Corina Satler[3], Ana Garcia[1,4], Rosângela C. Rodrigues[1], Soraya L. de Sá Canabarro[1] and Carlos Tomaz[5]**

[1] Laboratory of Neurosciences and Behavior, Department of Physiological Sciences, University of Brasilia, Brasilia, Brazil, [2] Institute of Cognitive Neuroscience, University College London, London, United Kingdom, [3] Faculty of Ceilandia, University of Brasilia, Brasilia, Brazil, [4] Euro-American University Center (UNIEURO), Brasilia, Brazil, [5] Neuroscience Research Program, CEUMA University, São Luís, Brazil

Many studies have investigated the influence of emotion on memory processes across the human lifespan. Some results have shown older adults (OA) performing better with positive stimuli, some with negative items, whereas some found no impact of emotional valence. Here we tested, in two independent studies, how younger adults (YA) and OA would perform in a visuospatial working memory (VSWM) task with positive, negative, and neutral images. The task consisted of identifying the new location of a stimulus in a crescent set of identical stimuli presented in different locations in a touch-screen monitor. In other words, participants should memorize the locations previously occupied to identify the new location. For each trial, the number of occupied locations increased until 8 or until a mistake was made. In study 1, 56 YA and 38 OA completed the task using images from the International Affective Picture System (IAPS). Results showed that, although YA outperformed OA, no effects of emotion were found. In study 2, 26 YA and 25 OA were tested using facial expressions as stimuli. Data from this study showed that negative faces facilitated performance and this effect did not differ between age groups. No differences were found between men and women. Taken together, our findings suggest that YA and OA's VSWM can be influenced by the emotional valence of the information, though this effect was present only for facial stimuli. Presumably, this may have happened due to the social and biological importance of such stimuli, which are more effective in transmitting emotions than IAPS images. Critically, our results also indicate that the mixed findings in the literature about the influence of aging on the interactions between memory and emotion may be caused by the use of different stimuli and methods. This possibility should be kept in mind in future studies about memory and emotion across the lifespan.

Keywords: aging, visuospatial working memory, emotion, facial stimuli, IAPS

OPEN ACCESS

Edited by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico

Reviewed by:
Rocco Palumbo, Harvard Medical School, United States
Assunta Pompili, University of L'Aquila, Italy
Peter Lewinski, Kozminski University, Poland

***Correspondence:**
Carlos Tomaz
ctomaz@ceuma.br

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *26 June 2017*
Accepted: *07 September 2017*
Published: *26 September 2017*

Citation:
Belham FS, Tavares MCH, Satler C, Garcia A, Rodrigues RC, Canabarro SLS and Tomaz C (2017) Negative Facial Expressions – But Not Visual Scenes – Enhance Human Working Memory in Younger and Older Participants. Front. Pharmacol. 8:668. doi: 10.3389/fphar.2017.00668

INTRODUCTION

Visuospatial working memory (VSWM), one of the storage subsystems of working memory (Baddeley and Hitch, 1974, 1994), has been found to be impacted by cognitive aging (Fabiani et al., 2015; Cabeza et al., 2016). Hale and colleagues, for instance, conducted a series of studies comparing age-related differences between visuospatial and verbal working memory

(Jenkins et al., 2000; Myerson et al., 2003; Hale et al., 2011) and found stronger differences between younger (YA) and older adults (OA) in the visuospatial domain, suggesting that the latter is severely affected by aging (see also Bopp and Verhaeghen, 2007). Nevertheless, the effects of emotional processing in the age-related decline in VSWM have yet to be understood. In other words, although it is known that the modulatory effect of emotion on memory changes across the lifespan (Scheibe and Carstensen, 2010), it is not clear how these alterations occur in VSWM.

A common idea in the literature about how aging affects memory and emotion interactions is the Positivity Effect, which refers to how OA's memory is biased toward positive events or stimuli (Carstensen and Mikels, 2005; Mikels et al., 2005; Petrican et al., 2008). A proposal by Mammarella et al. (2016a) offers a biological explanation for this effect, based on the modulatory influences of noradrenaline on emotional memory (Tully and Bolshakov, 2010). According to Mammarella et al. (2016a), recent evidence points to noradrenaline being linked to behavioral changes related to motivation, reward, and stimuli salience (Bouret and Richmond, 2015; Mather et al., 2015). Another line of evidence suggests an increase in activity of the noradrenergic system in aging (Seals and Esler, 2000), which would increase the effects of this component on OA's emotional memory. Additionally, if aging brings a shift in a person's goals, motivation, and interests, distinct stimuli may become more or less salient and rewarding. That is, if OA are more focused on emotionally meaningful and positive experiences, these will possibly be differently affected by noradrenaline compared to YA, leading to the Positivity Effect. In fact, such age-related change in goals, motivations, and interests is compatible with another proposal, named the Socioemotional Selectivity Theory (Carstensen et al., 1999). This theory states that one's goals depend on their temporal context. Whereas YA perceive their remaining lifetime as long, OA tend to focus their cognitive resources on the pursuit of emotionally meaningful experiences. Joining Mammarella et al. (2016a)'s and Carstensen et al. (1999)'s proposals, OA's focus on positive events may make these events more salient and differentially modulated by noradrenaline, enhancing their memorization.

Recent studies have supported the presence of a Positivity Bias in OA using different types of stimuli, including images from the International Affective Picture System (IAPS) (Lang et al., 1997;Mammarella et al., 2016b; Kan et al., 2017) and facial stimuli (Castel et al., 2016; Sava et al., 2017). Nevertheless, it is necessary to point out that some studies did not find a Positivity Effect. Those studies found a Negative Bias instead, with both YA and OA having better memory performances for negative items (Gruhn et al., 2005; Thomas and Hasher, 2006; Satler and Tomaz, 2011; Belham et al., 2013; Foster et al., 2013). This may happen because negative stimuli require prompter responses from an evolutionary point of view (Rozin and Royzman, 2001; Galli et al., 2011). Additionally, some studies simply have found no effect of emotion on memory whatsoever (Denburg et al., 2003; Garcia et al., 2011).

Regarding working memory specifically, recent studies have found mixed effects of emotion on OA's performance. For example, Ziaei et al. (2015, 2017) found no effects of emotional valence when younger and older participants had to indicate if an IAPS image had been presented during the past three trials. Bermudez and Souza (2016), however, asked participants to indicate the six positions previously occupied by IAPS images in an array of 16 images. Valence did not affect YA's behavior, but OA performed significantly worse with negative images. Mammarella et al. (2013) used a working memory task with emotional words and found that positive valence had a beneficial effect in OA but not in YA. Using the same task, Borella et al. (2014) found that both age groups performed better with negative words, although this result seemed to be influenced by individual differences. In a different study also using words, Truong and Yang (2014) found that negative and positive valence equally facilitated performance and the effects did not between the two age groups.

These mixed findings, together with the lack of knowledge about how OA's VSWM is influenced by emotions, lead to the necessity of more studies. Here we aimed to investigate how YA and OA would perform in a VSWM test with negative, positive, and neutral stimuli. Younger and older participants responded the Spatial Delayed Recognition Span Task (SDRST) (Satler et al., 2015), in which identical stimuli are presented in different locations in a crescent set of up to eight locations. This task was chosen because it requires that participants keep the initial spatial locations in mind to be able to identify the new location within each trial. The locations are changed in each new trial and participants must update the information in their VSWM. This task has previously been used with YA and OA (Satler et al., 2015). We predicted that emotional valence would facilitate performance in both age groups. However, as detailed in the previous paragraph, the working memory literature shows mixed results in terms of how the age groups' performance is modulated by the emotional valence of the stimuli. Thus, we did not have a strong prediction as to which valence (negative or positive) would have a larger influence in the current study. We report two independent studies using the same SDRST. Study 1 used negative, positive, and neutral images from the IAPS. In study 2, the stimuli were composed of angry, happy, and neutral facial expressions. We were interested in how accuracy in this VSWM task would be affected by the different valences displayed by the stimuli on the screen.

STUDY 1

Materials and Methods

Participants and Stimuli

Study 1 included 56 YA recruited from the university's undergraduate programs (30 women; mean age 21.38 ± 2.90; at least 13 years of formal education) and 39 healthy OA (24 women; mean age 71.10 ± 6.72; at least 13 years of formal education) recruited from the Geriatric Medical Center, University Hospital of Brasilia. All were right-handed volunteers with no history of neurological or psychiatric episodes and no recent use of psychotropic medication, as evaluated by a detailed anamnesis. The eligibility criteria also included no consumption of alcohol or drugs in the 24 h prior to testing. Participants were vaguely informed about the aims of the study, and signed a written

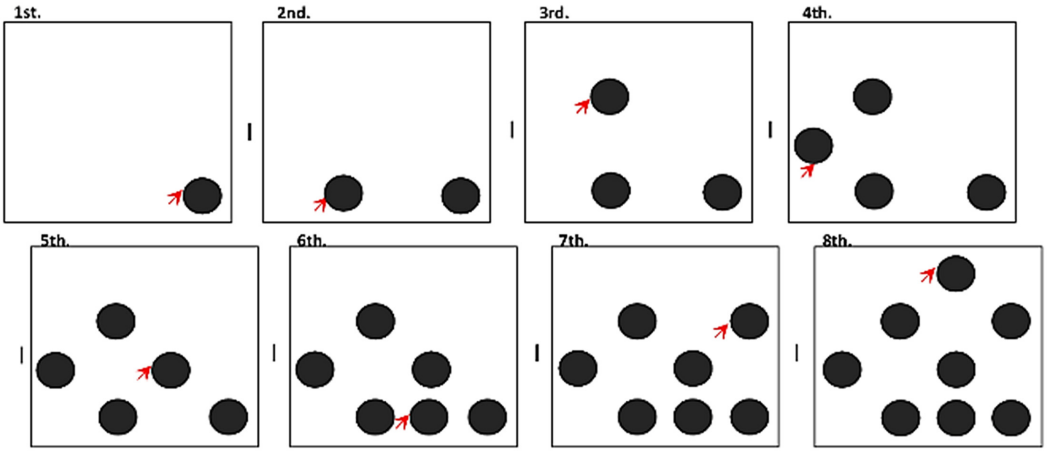

FIGURE 1 | Experimental design for the Spatial Delayed Recognition Span Task (SDRST). Participants had to memorize the occupied locations and identify the new location within each trial up to a maximum of eight locations. The task was conducted on a touch-screen monitor.

informed consent in accordance with the ethical guidelines for research with human subjects (196/96 CNS/MS Resolution). The study was approved by the Human Subjects Ethics Committee of the Health Sciences Faculty of the University of Brasilia (CEP-FS160/08 and CEP-FM064/2007). All participants had normal or corrected-to-normal vision and hearing. OA scored at least 24 on the Mini-mental State Examination (Folstein et al., 1975) and less than 9 on the Geriatric Depression Scale (Yesavage and Sheikh, 1986).

The stimuli material used in study 1 were composed of emotional pictures selected from the IAPS (Lang et al., 1997) and could depict objects and scenes. Three positive images (slides number: 5750, 5030, 1660; valence: 6.60, 6.51, 6.49; and arousal: 3.14, 2.74, 4.57, respectively), four negative images (slides number: 3000, 3120, 3130, 3030; valence: 1.41, 1.56, 1.58, 1.91; and arousal: 7.26, 6.84, 6.97, 6.76, respectively), and two neutral images (slides number: 5510, 7010; valence: 5.15, 4.94; and arousal: 2.82, 1.76, respectively) were selected. No facial images were selected for study 1. Each participant responded to nine trials, one for each image, presented in a pseudo-randomized order. Computer software registered correct and incorrect responses for each given answer. The time of execution of the task varied according to each participant's response time, but the full procedure did not last more than 2 h.

The Spatial Delayed Recognition Span Task (SDRST)

The task was a computer-based version (Delphi language, computational program TREA) of the SDRST, which measures participants' working memory (Lacreuse et al., 2005). The task is presented to YA and OA on a touch-screen monitor (LG Studio Works 440, Microtouch, 17′) positioned within arm's reach. The computer-based SDRST has been successfully used with different populations and stimuli by our group (Satler et al., 2015). In this task, participants must discriminate a novel location of a stimulus among an increasing array of identical stimuli presented sequentially in various locations within the same trial. At the beginning of each trial, one stimulus is presented at random in 1 of the 16 possible locations on the screen. Participants must touch it. After a pre-determined delay, that stimulus re-appears in the same position and another identical stimulus appears in a new position. Participants must touch the stimulus presented in the new location. Every time a correct response is made, a new stimulus is added to the array. This goes on until the maximum of eight stimuli or until a mistake is made. In both cases, a new trial with a different stimulus begins. Correct answers led to the emission of an acute auditory feedback signal; wrong answers led to a bass auditory signal. Stimuli within a single trial were identical and did not repeat in two consecutive trials.

The stimuli were presented for a period of up to 5 s (1 s for YA, 5 s for OA) (**Figure 1**). Before the beginning of the task, every participant received written and oral instructions and completed a practice session. The practice session used geometrical shapes as stimuli and was conducted in the same fashion as the main task. The practice session was concluded when participants correctly answered two consecutive complete trials (eight stimuli per trial) or after 20 trials.

Statistical Analyses

Accuracy was calculated as the mean of correct choices before a mistake for all the trials of each emotional valence. A mixed-design ANOVA was run (SPSS v. 18.00; SPSS, Inc., Chicago, IL, United States, 2009) with age (YA or OA) as a between-subjects factor, and emotional valence (neutral, positive, and negative) as a within-subjects factor. Significance was defined as a p-value < 0.05.

Results

Mean accuracy for YA and OA can be seen in **Table 1**. No differences were found between men and women ($p > 0.205$). Accuracy in this task was not influenced by the emotional valence of the IAPS image ($F_{(2,186)} = 0.293$, $p = 0.706$, $\eta_p^2 = 0.004$). However, YA outperformed OA ($F_{(1,93)} = 65.217$, $p < 0.001$, $\eta_p^2 = 0.412$). There was no significant interaction between the factors ($F_{(2,186)} = 0.723$, $p = 0.487$, $\eta_p^2 = 0.008$) (**Figure 2**).

TABLE 1 | Accuracy during the Spatial Delayed Recognition Span Task (SDRST) for Younger adults and Older adults with positive, neutral, and negative IAPS images.

	Positive	Neutral	Negative	Average
Younger adults ($n = 56$)	7.52 (0.75)	7.27 (1.23)	7.31 (1.10)	7.36 (0.77)
Older adults ($n = 39$)	5.61 (1.67)	5.64 (1.54)	5.69 (1.34)	5.65 (1.29)
Average	6.74 (1.53)	6.60 (1.58)	6.64 (1.44)	

Mean (SD) of a maximum of 8.

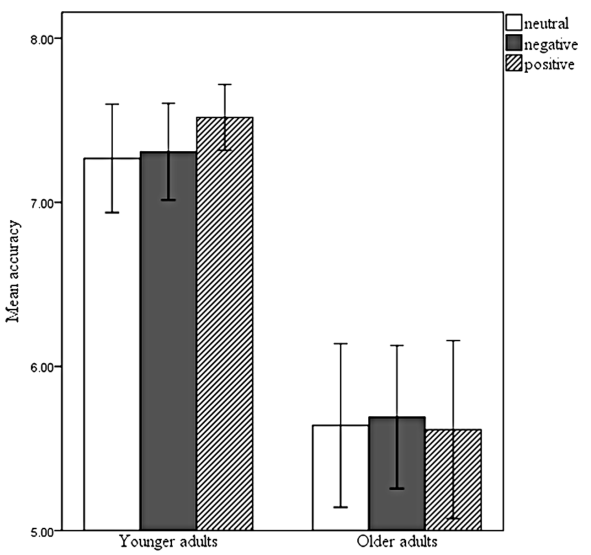

FIGURE 2 | Mean accuracy for younger (YA) and older adults (OA) responding to the SDRST with neutral, negative, and positive IAPS images. Error bars represent confidence intervals (95%). YA outperformed OA ($p < 0.001$). There was no significant influence of emotional valence ($p = 0.706$).

Discussion

The age-related difference in performance found in study 1 is in line with previous research, suggesting that healthy aging is related to deficits in various cognitive domains, including VSWM. For instance, Jenkins et al. (2000) tested YA and OA in visuospatial speed, memory, and learning tasks and discovered an age-related decline on all three types of processing.

The absence of a valence effect on memory performance was surprising and unexpected. As mentioned before, several studies support the existence of a Negativity Bias in YA and a Positivity Effect in OA. Nevertheless, the current results are in line with other investigations reporting no valence effects on memory performance, such as Denburg et al. (2003) and Garcia et al. (2011). The present results are also in line with at least other two studies who found no effects of emotion on YA and OA's WM for IAPS images (Ziaei et al., 2015, 2017).

One possibility is that the non-significant effect of valence in the current study was caused by the fact that the IAPS images are not a straightforward way of displaying emotions, being sometimes too complex or rich in visual information (Britton et al., 2008). By contrast, facial photographs with different emotional expressions are considered by many studies as one of the most important and direct ways of externalizing emotions (Hess et al., 1997; Nahm et al., 1997). For example, Isaacowitz et al. (2007) investigated age-related differences in the recognition of emotions from lexical stimuli (sentences describing emotional situations) and facial expressions. They found an interaction between age group and task type, indicating that facial stimuli elicited significantly less age differences than did the lexical stimuli. Some researchers, on the other hand, propose that aging brings deficits in the recognition of negative facial expressions, but not of positive ones (Orgeta and Phillips, 2007; Kellough and Knight, 2012). Moreover, Altamura et al. (2016) found that OA are quicker in identifying a positive facial expression compared to a negative one. Thus, we decided to conduct a second study using photographs of facial expressions as stimuli. Due to the social and biological relevance of facial stimuli, we predicted that study 2 would lead to different results from study 1, with emotional valence influencing memory performance of the two age groups.

STUDY 2

Materials and Methods

Inclusion and exclusion criteria were the same as used in Study 1. Twenty-six YA recruited from the university's undergraduate programs (13 women; mean age 21.31 ± 2.05 years; at least 14 years of formal education) and 25 healthy OA (11 women; mean age 69.92 ± 6.41 years; at least 13 years of formal education) recruited from the Geriatric Medical Center, University Hospital of Brasilia took part in this study. This study was approved by the Human Subjects Ethics Committee of the Health Sciences Faculty of the University of Brasilia (CEP-FS 097/11). For this study, all participants scored more than 24 on the Mini-mental State Examination (Folstein et al., 1975) and less than 9 on the Geriatric Depression Scale (Yesavage and Sheikh, 1986).

The same SDRST was conducted (**Figure 1**) using colored photographs (4 cm × 4 cm) of adult models displaying different facial expressions, manipulated to only show the face, with no interference from hair or other body parts. The facial data set was provided to our group by Dr. Hisao Nishijo from the University of Toyama, Japan, and has been used in previous studies from that lab (Hori et al., 2005). Seven images of happiness (positive valence), two images of anger (negative valence), and two neutral expressions were chosen from the original data set based on a pilot study where 30 YA and OA (who did not participate in the main study) identified the emotional expression of several faces displayed on the screen. Only images that elicited a correct classification rate of over 90% were chosen. Each participant responded to one block of each emotional valence. Each block contained 10 trials and two identical faces were never presented in two consecutive trials. Images were presented for 3 s.

Results

Mean accuracy for YA and OA can be seen in **Table 2**. No differences were found between men and women ($p > 0.107$). Accuracy was influenced by the valence of the facial expression ($F_{(2,98)} = 6.024$, $p = 0.003$, $\eta_p^2 = 0.109$). Pairwise comparisons adjusted with the Bonferroni correction showed that negative

items elicited much better performance than positive items ($p = 0.002$) and a marginally better performance than neutral items ($p = 0.084$). Neutral items did not differ from positive items ($p = 0.894$). YA performed better than OA ($F_{(1,49)} = 40.198$, $p < 0.001$, $\eta_p^2 = 0.451$). There was no significant interaction between the factors ($F_{(2,98)} = 0.351$, $p = 0.705$, $\eta_p^2 = 0.007$) (**Figure 3**).

Discussion

In study 2, YA again performed better than OA, as expected. However, this time valence influenced memory accuracy, with negative faces eliciting a better performance than positive faces and neutral faces (marginally). This is called the Negativity Bias. It states that, due to their larger influence on the adaptive value of an individual, negative events are more efficiently remembered (Rozin and Royzman, 2001). Negative stimuli also attract more attention to their location and generate a more prompt behavioral response because they indicate places to be avoided, possibility of contamination, and imminent threats from others (reviewed by Palermo and Rhodes, 2007). Being in a negative emotional state has also been shown to improve cognition (Gray et al., 2002). Importantly, study 2 revealed no interaction between age and emotion, suggesting that negative emotion can benefit memory performance of both YA and OA, as found in previous studies (Gruhn et al., 2005; Thomas and Hasher, 2006; Foster et al., 2013). In other words, the results of the current study suggest that YA and OA did not differ in how their VSWM is influenced by the emotional valence displayed in facial stimuli.

The absence of the Positivity Effect in OA may have been due to the difficulty of the task. Two studies (Mather and Knight, 2005; Knight et al., 2007) presented participants with either full-attention tasks or divided-attention tasks and showed that, when the task is more cognitively demanding, the tendency of OA to favor positive over negative images is eliminated. It is also possible that the Positivity Effect is not as universal as originally thought, since the current study and others studies mentioned throughout this paper have failed to find it.

On a different note, Lewinski (2015) argues that humans only rate neutral facial expressions as neutral in 60% of the cases. This result does not suggest that the neutral faces used in the current study were inappropriate. Rather, it raises the possibility that the 30 pilot participants failed to recognize other faces as neutral. Future research may take advantage of automated facial coding software to aid in stimuli selection.

TABLE 2 | Accuracy during the SDRST for Younger adults and Older adults with positive, neutral, and negative facial stimuli.

	Positive	Neutral	Negative	Average
Younger adults ($n = 26$)	7.26 (0.72)	7.42 (0.70)	7.56 (0.45)	7.41 (0.54)
Older adults ($n = 25$)	5.84 (1.02)	5.90 (1.22)	6.19 (1.06)	5.98 (1.01)
Average	6.57 (1.13)	6.67 (1.24)	6.89 (1.06)	

Mean (SD) of a maximum of 8.

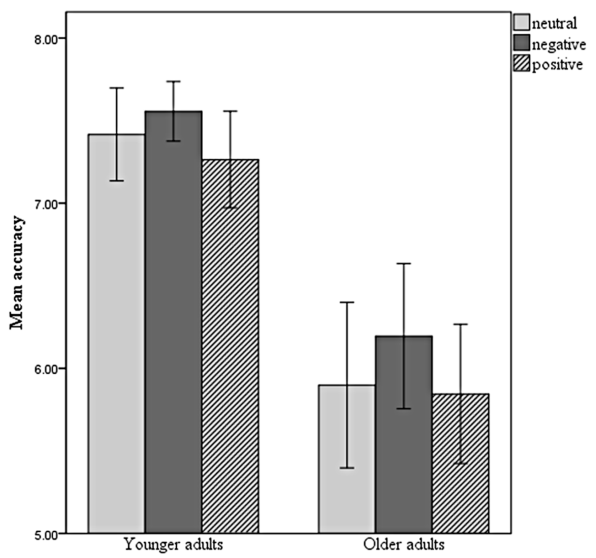

FIGURE 3 | Mean accuracy for YA and OA responding to the SDRST with neutral, negative, and positive facial photographs. Error bars represent confidence intervals (95%). YA outperformed OA ($p < 0.001$). Performance with negative images was higher than with positive images ($p = 0.002$).

GENERAL DISCUSSION: IAPS × FACES

The present research explored the responses of YA and OA performing a VSWM task that required the processing of emotional stimuli. The main goal was to investigate age-related differences in the emotional modulation of VSWM. Study 1 used positive, negative, and neutral IAPS pictures, whereas study 2 presented participants with happy, angry, and neutral facial expressions. As expected, in both studies YA showed higher accuracy than OA. Interestingly, however, only in study 2 emotion influenced memory, with negative faces leading to a better performance. The results were not influenced by whether participants were male or female, suggesting no sex-related differences in the relationship between VSWM and emotion.

A few possible explanations for the different results between the two studies can be raised, based on how faces differ from other visual stimuli. First, faces are one of the most important and straightforward ways of externalizing emotions (Hess et al., 1997). When directly compared with IAPS images, faces are less ambiguous and more familiar, which enhances the efficiency of their processing (Öhman et al., 2001; Britton et al., 2008; Ekman and Cordaro, 2011). Second, studies have demonstrated that faces are more quickly detected than other types of stimuli (Palermo and Rhodes, 2007), partially due to the Fusiform Face Area (Kanwisher et al., 1997). Liu et al. (2002) showed that a stimulus is categorized as a face in extricate areas of the brain no longer than 100 ms after the stimulus presentation, which does not happen for other images (e.g., houses). The fact that facial stimuli are more quickly identified and do not require additional cognitive load (Britton et al., 2008) may have allowed for a faster processing of the emotional information in those stimuli and, in consequence, strengthened their influence on VSWM when compared to IAPS images. Finally, IAPS images

are more complex and more distinct between each other than faces. These characteristics induce a slower habituation to novelty and demand more sustained attention during the task (Knight et al., 2007; Britton et al., 2008). Previous research has shown that the effects of emotion on memory are reduced when participants are instructed to pay extra attention to stimuli (Talmi et al., 2008). Thus, the additional attentional resources required by the IAPS images may have reduced the influence of their emotional content on memory, when compared to emotional faces. All this evidence supports the conclusion that faces are more efficient in transmitting emotional valence and, thus, influencing working memory, than other types of stimuli such as contextual pictures.

Some limitations of the current study should be addressed. Our experiments were not designed to directly test the influence of the type of stimuli on memory and emotion interaction in OA. Study 1 was designed to investigate VSWM for emotional images and study 2 was developed in consequence of the results from the first study. Also, in study 1, the stimuli presentation times differed between the two age groups due to OA being slower in their movements and less familiar with the use of the computer. In study 2 we used the same stimulus duration for both age groups. Because of this, a direct comparison between IAPS and facial stimuli in the same study should be conducted to strengthen our conclusions. The facial stimuli used in our studies showed pictures of adult models, but not of OA. This means that our older participants were responding to out-group faces, which could lead to different cognitive processing (Samanez-Larkin and Carstensen, 2011). However, previous research has demonstrated that both YA and OA are faster and more accurate in identifying emotional expressions in younger than in older faces (Ebner et al., 2012). This finding, and the fact that we found no interactions between emotion and aging, supports the conclusion that the facial stimuli used here were adequate, though future research could directly repeat our experiments using older faces. It is important to highlight that the use of only anger as the negative emotional expression was dictated by the pilot study described in the "Materials and Methods" section of study 2. However, we are aware that the processing of negative valence may differ due to the specific emotion being tested (Phan et al., 2002). Thus, it is important that future studies repeat our experiments with a larger variety of facial expressions (e.g., sadness and disgust) within the same emotional valence.

CONCLUSION

Our findings suggest that the emotional modulation of VSWM is influenced by the type of information to be remembered. This effect seems to be present for YA and for OA. Taken together, our results contribute to the understanding of information processing in YA and OA and to the characterization of cognition across the human lifespan. This knowledge may explain the inconsistent literature findings about the interplay between memory, emotion, and aging, and lead to the development of better methodological approaches when studying these topics.

AUTHOR CONTRIBUTIONS

FB, MT, CS, AG, and CT: designed study. FB, CS, and AG: collected data. FB, MT, CS, AG, and CT: analyzed data. FB, MT, CS, AG, RR, SC, and CT: interpreted data. FB, MT, CS, AG, RR, SC, and CT: wrote paper.

FUNDING

This work was partially supported by a FAPEMA grant to CT. FB and CS were recipients of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) scholarships when the studies were conducted. FB is now a recipient of a scholarship by the Capes Foundation within the Ministry of Education, Brazil (grant no. BEX 99999.006087/2013-02). MT is a fellowship recipient of CNPq (PQ 2, 2015, 311582/2015). SC is a recipient of a scholarship by CNPq.

ACKNOWLEDGMENT

We thank Prof. Dr. Concepta Margareth MacManus Pimentel for the English revision of this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 03 August 2017
doi: 10.3389/fphar.2017.00515

Zebrafish as a Model for Epilepsy-Induced Cognitive Dysfunction: A Pharmacological, Biochemical and Behavioral Approach

*Uday P. Kundap, Yatinesh Kumari, Iekhsan Othman and Mohd. Farooq Shaikh**

Neuropharmacology Research Laboratory, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Selangor, Malaysia

Epilepsy is a neuronal disorder allied with distinct neurological and behavioral alterations characterized by recurrent spontaneous epileptic seizures. Impairment of the cognitive performances such as learning and memory is frequently observed in epileptic patients. Anti-epileptic drugs (AEDs) are efficient to the majority of patients. However, 30% of this population seems to be refractory to the drug treatment. These patients are not seizure-free and frequently they show impaired cognitive functions. Unfortunately, as a side effect, some AEDs could contribute to such impairment. The major problem associated with conducting studies on epilepsy-related cognitive function is the lack of easy, rapid, specific and sensitive *in vivo* testing models. However, by using a number of different techniques and parameters in the zebrafish, we can incorporate the unique feature of specific disorder to study the molecular and behavior basis of this disease. In the view of current literature, the goal of the study was to develop a zebrafish model of epilepsy induced cognitive dysfunction. In this study, the effect of AEDs on locomotor activity and seizure-like behavior was tested against the pentylenetetrazole (PTZ) induced seizures in zebrafish and epilepsy associated cognitive dysfunction was determined using T-maze test followed by neurotransmitter estimation and gene expression analysis. It was observed that all the AEDs significantly reversed PTZ induced seizure in zebrafish, but had a negative impact on cognitive functions of zebrafish. AEDs were found to modulate neurotransmitter levels, especially GABA, glutamate, and acetylcholine and gene expression in the drug treated zebrafish brains. Therefore, combination of behavioral, neurochemical and genenetic information, makes this model a useful tool for future research and discovery of newer and safer AEDs.

Keywords: zebrafish model development, epilepsy, anti-epileptic drugs, cognitive dysfunction, T-maze

OPEN ACCESS

Edited by:
Assunta Pompili,
University of L'Aquila, Italy

Reviewed by:
Hiram Luna-Munguia,
University of Michigan, United States
Massimo Grilli,
Università di Genova, Italy

***Correspondence:**
Mohd. Farooq Shaikh
farooq.shaikh@monash.edu

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *21 April 2017*
Accepted: *21 July 2017*
Published: *03 August 2017*

Citation:
Kundap UP, Kumari Y, Othman I and Shaikh MF (2017) Zebrafish as a Model for Epilepsy-Induced Cognitive Dysfunction: A Pharmacological, Biochemical and Behavioral Approach. Front. Pharmacol. 8:515. doi: 10.3389/fphar.2017.00515

INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by unpredictable seizures, which may differ from a brief lapse of attention and muscle cramps to severe and long-lasting convulsions (Zashikhina, 2014). It has a poorly understood pathologic mechanism and is an intricate brain disorder with numerous fundamental causes (Galanopoulou et al., 2012). The multifactorial nature

of epilepsy needs to be taken into consideration when developing therapeutic strategies overcoming selected mechanisms. Its clinical management is predominantly based on the administration of anti-epileptic drugs (AEDs) aiming to suppress the seizure activity. Although more than 20 AEDs are available, nearly 30% of the epileptic patients are refractory to drug-treatment. The AEDs are used to modify the processes involved in epileptogenesis and promote inhibition over excitation and thus prevent epileptic seizure (White et al., 2007). The AEDs act by several mechanism but the major mechanisms of action, include γ-aminobutyric acid (GABA) enhancers, glutamate blockers, calcium current inhibitors and sodium channel blockers (Czapinski et al., 2005). AEDs enhance inhibitory neurotransmission or suppress neuronal excitability. (Aldenkamp et al., 2003). Owing to the advantages of wide availability, lower cost and long-term experience older AEDs are still been prescribed, but greater effects are often exhibited by older AEDs (Eddy et al., 2011). Newer agents which are currently established have differences in their pharmacokinetic properties, mode of pharmacological action (Bootsma et al., 2009).

Cognitive impairment is a common comorbidity in multiple brain disorders like epilepsy, Alzheimer's disease (AD), schizophrenia, Huntington's disease (HD) and autism (Vingerhoets, 2006). Brain areas affected due to electric discharge in epilepsy are temporal lobe, hippocampus, medial frontal brain regions, bilateral superior temporal and subthalamus brain regions in epileptic patients (Laufs et al., 2007). It is better agrued that seizure-induced neuronal modeling during epilepsy and recurrent seizures can cause continuous neuronal reorganization (Pitkänen and Sutula, 2002). As temperol lobes and hippocampus is associated with memory formation, it is not shocking that epilepsy in such area can cause memory dysfunctioning (Helmstaedter and Kockelmann, 2006). Neurotransmitters are important in maintaining the normal brain functions and also known to be modulated during a brain insult. Alteration of neurotransmitters has been found to be closely associated with epilepsy. Some important neurotransmitters known to paly a significant role in epilepsy and cognition are GABA, glutamate and acetylcholine (Sancheti et al., 2013). GABA is an inhibitory transmitter and known for its role in suppressing epilepsy (Rico et al., 2011). Glutamate is an excitary chemical which causes neuronal death and is associated with glutamate neuro toxicity in epilepsy (Ozawa et al., 1998). Acetylcholine (ACh) plays the key role in modulating glutamate release and memory formation. It is reported that AEDs reduce neuronal irritability but also may impair neuronal excitability, neurotransmitter release, enzymes, genes and factors critical for information processing and memory (Hamed, 2009). As per a clinical study done at Columbia University, New York, NY, United States over all AEDs related cognitive side effect (CSEs) was found to be 12.8%. Drug specific CSEs for gabapentin (7.3%), oxcarbazepine (11.6%), phenytoin (12.9%). From the study, it was concluded that patient-reported CSEs are most common with topiramate (TPM), followed by zonisamide, phenytoin, and oxcarbazepine (Arif et al., 2009).

Zebrafish has a complex nervous system capable of sophisticated behaviors and susceptible to seizures. A full range of mature behavior can be studied in adult zebrafish which makes them particularly desirable for model development. In last few years, the use of zebrafish has gained popularity as an alternative to rodents and other experimental animals for the study of the molecular mechanisms underlying cognition deficit and for the screening of potential therapeutic compounds (Kalueff et al., 2014). There is much scientific evidence illustrating the benefits of animals, such as zebrafish, as a replacement and better animal model for drug discovery (**Figure 1**). Genetic structure of zebrafish is similar to human. Around 70 percent of genes are shared with humans and about 84 percent of genes known to human disease are also expressed in zebrafish (Norton and Bally-Cuif, 2010). The overall cost of the studies which involves large animals are expensive and laborious. The small animal or rodent models are preferred to study safety profile of a drug and for testing of the hypothesis (Jucker, 2010). The non-human primate and rodents models are similar to humans in terms of their anatomy, physiology and behavior, but they are not so common due to ethical and economical concerns (Jucker, 2010). The cost and time required to carry out the study in zebrafish model is less as compared to rodents (Mussulini et al., 2013). For studying various brain disorders, zebrafish (*Danio rerio*) is quickly mounting as a promising model creature (Kundap et al., 2016). It is important to minimize animal distress by using the least sensitive organism possible to answer the question at hand. One of the biggest challenges in conducting research on epilepsy and cognitive function is the absence of a precise, sensitive, and reproducible disease model (Vining, 1987).

Pentylenetetrazole (PTZ) is used in zebrafish to induce epileptic seizure-like condition and to study the mechanisms of seizures (Desmond et al., 2012). γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the brain, PTZ exerts its convulsive effect by inhibiting the activity of GABA at $GABA_A$ receptors (Shaikh et al., 2013). There are several reports which provide strong evidence on the usefulness of zebrafish model for studying cognitive functions. Investigators used active avoidance paradigm to discover a high impact about learning and memory in zebrafish. In this method zebrafish are trained to associate light with shock stimulus in a fish shuttle-box (Xu and Goetz, 2012). Zebrafish has natural color preference choice and it has received little attention in past few research. Natural color preference concerning a precise color may lead to changes in learning, decision making, memory and visual discrimination (Avdesh et al., 2010). The zebrafish is becoming an popular model increasingly for investigating treatment method and understanding the process behind memory problems in AD (Newman et al., 2014). The T-maze is a forthright method to test the learning skill, long- and short-term memory, and memory plasticity in zebrafish (Vignet et al., 2013). The T-maze has been most comprehensively used to examine specific features of spatial working and learning memory (Wenk, 2001).

In the view of current literature, the prime goal of the study was to develop a zebrafish model of epilepsy induced cognitive dysfunction and simulate the clinical condition which shows that both epilepsy and AEDs negatively affect the cognitive functions. This zebrafish model will serve as an important tool

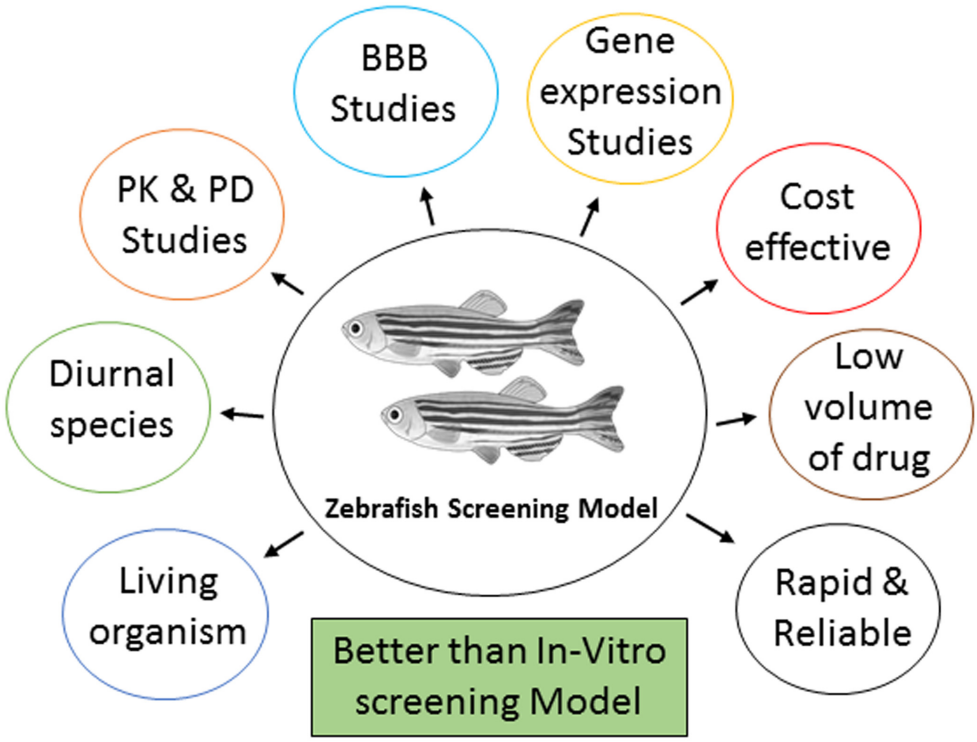

FIGURE 1 | The significance of using zebrafish as a screening model.

for the development and screening of newer and safer anti-epileptic drugs with intact memory functions. As epilepsy involve important biological and physiological mechanisms, studying zebrafish behavior, neurotransmitters level and gene expression are of great significance and key parameters in the development of an impressive animal model.

MATERIALS AND METHODS

Chemicals and Equipment

Glutamic acid (Glu), γ-aminobutyric acid (GABA), Acetylcholine (ACh) and Pentylenetetrazole (PTZ) were from Sigma–Aldrich (United States). All the standard AEDs such as Phenytoin (PHY), Oxcarbazepin (OXC), Gabapentin (GBP), Diazepam (DZP), Rivastigmine (RSV) were from Sigma–Aldrich (United States). Dimethyl sulfoxide (DMSO) was purchased from Vivantis Inc (United States). Sony video recorder, Smart V3.0.05 tracking software (Pan Lab, Harvard apparatus), Agilent 1290 Infinity UHPLC, coupled with Agilent 6410 Triple, Quad LC/MS, Milli-Q system from Millipore (Bedford, MA, United States), Applied Biosystems StepOnePlus™ Real-Time PCR Systems.

Animal Care

Adult zebrafish (*Danio rerio*; 3–4 months-old) of heterogeneous strain wild-type stock (standard short-fin phenotype) were obtained from a Akarium Batukarang, Subang Jaya, Malaysia. All fish were housed in the animal facility of Monash University Malaysia under standard husbandry conditions. Fish were maintained under temperature 28°C ± 2°C, pH between 6.8 and 7.1 and light intensity 250 lux with 14 h light: 10 h dark regime (light onset: 8am; light offset: 10pm). Fish were fed with TetraMin® Tropical Flake and live Artemia from Bio-Marine Brand (Aquafauna, Inc. United States) three times a day to ensure a constant source of nourishment with *ad libitum* feeding. Standard zebrafish tank which are equipped with circulating water system with constant aeration having tank dimensions of (36 cm × 26 cm × 22 cm) were used as shown in **Figure 2**. Group housing (10–12 fishes/tank), males and females having seperate housing arrangement. All the animal experimentations were approved by Monash Animal Research Platform (MARP), Australia.

Drug Treatment and Groups

Adult male zebrafish (*Danio rerio*) were used. PHY, OXC, GBP, DZP, RSV and PTZ were dissolved in 10% DMSO. Three months old fish were selected with a weight range of 0.5–0.6 g. Animal were divided into following groups, Group I: Vehicle control (10% DMSO); Group II: Pentylenetetrazole (PTZ-Negative control group); Group III: Phenytoin 80 mg/kg (PHY) + PTZ (170 mg/kg); Group IV: Oxcarbazepin 80 mg/kg (OXC) + PTZ (170 mg/kg); Group V: Gabapentin 800 mg/kg (GBP) + PTZ (170 mg/kg); Group VI: Diazepam 1.25 mg/kg (DZP) + PTZ (170 mg/kg); and Group VII: Rivastigmine 1.5 mg/kg (RSV) + PTZ (170 mg/kg). In the experiment 10–12 fisher group were used (**Figure 2**).

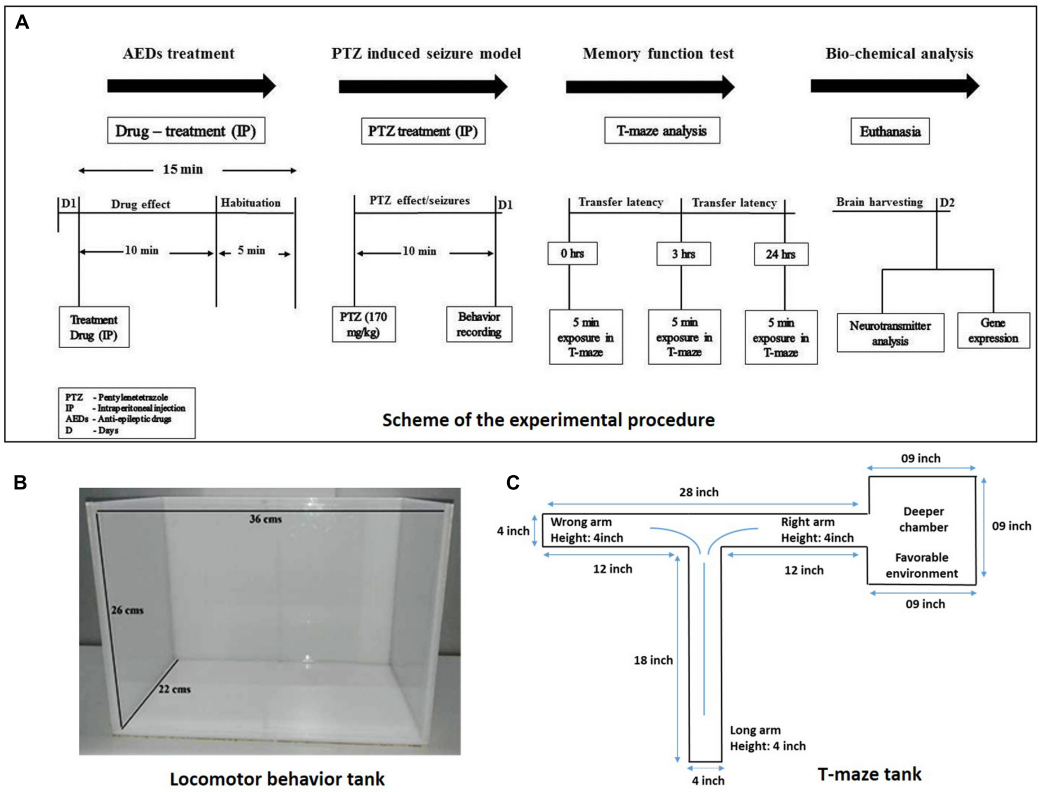

FIGURE 2 | Experimental procedure and experiment tanks used. **(A)** Represents scheme for the experiment procedure used to carry out the study. **(B)** Represent the tank used for the locomotor behavior study. **(C)** Illustrates the typical size and specification of T-maze apparatus.

Intraperitoneal Injection in Zebrafish

The vehicle, PTZ and AED treated groups were injected intraperitoneally (via posterior to the pelvic girdle into the abdominal cavity), using Hamilton syringe 10 μl (700 series, Hamilton 80400) (Stewart et al., 2011). The experiment was performed in a separate behavior room with constant room temperature of 28°C ± 2°C and humidity 50–60%. All the fish were acclimatized in the behavior room 2 h prior to experiment to avoid novel tank response. Precautions include using a small injection volume of 10 μl per gram of fish and a 35 gauge needle. Fish were restrained in water saturated sponge under benzocaine anesthesia to reduce the distress (Júnior et al., 2012). This technique of IP injection in zebrafish was found to be effective and did not cause any mortality throughout the experiment.

Fish was captured individually by fish holding net, then transfer into anesthesia solution (30 mg/L Benzocaine), Fish was taken out once anaesthesized and weighed to calculate the dose and the injection volume. A soft sponge of approximately 20 mm in height was saturated with water and set into 60 mm Petri dish. A cut of 10–15 mm deep was made on the sponge to restrain and hold the fish for injection. Intraperitoneal injection was made using a dissecting microscope by inserting the needle into the midline between the pelvic fins. Appropriate volume was injected according to the body weight. After injection, fish was immediately transferred to the tank.

PTZ-Induced Seizure Model

The fish were treated with vehicle/AEDs via intraperitoneal injection and then habituated for 15 min in the observation tank before administration of PTZ. The vehicle control group only received 10% DMSO and suspended for behavior recording. Fifteen minutes after vehicle/AEDs administration, the animals were exposed to PTZ (170 mg/kg, IP) which presented different seizure profiles, intensities and latency to reach the scores. The seizures last for 10 min post-PTZ administration, which gradually decreases with time.. Adult zebrafish were tested in an observation tank, where the seizure score were measured using a special scoring system as mentioned in **Table 1**. Under the directives of Monash Animal Research Platform (MARP)-Australia, the dose of PTZ was adjusted to 170 mg/kg in order

TABLE 1 | Seizure score analysis modified from Mussulini et al. (2013).

Seizure scores	Event of Behavior
Score 1	Short swim mainly at the bottom of the tank
Score 2	Increased swimming activity and high frequency of opercula movement.
Score 3	Burst swimming, left and right movements and erratic movements
Score 4	Circular movements

to get the highest seizure score of 4. Seizure score, seizure onset, total distance traveled, time spent in upper and the lower half of the tank were the parameters noted (Baraban et al., 2005).

T-maze Test

The T-maze is composed of one long (18′) and two short (12′) arms. One of the short arms is connected to a deeper square chamber (9 × 9′) which serve as a favorable environment for the fish (see **Figure 2**). Favorable environment is the chamber which is deeper and wider compare to other arms in T-maze and once fish finds it, they spend the majority of their time in it. The T-maze behavior test was performed in the behavior room of constant room temperature of 25°C – 26°C and humidity 50– 60%. Each fish was placed at the beginning of the long arm and the time required to reach deeper chamber was recorded in the 5 min exploration period. The time taken by the fish to travel into the deeper chamber was determined as transfer latency (TL). Transfer latencies were recorded at 0, 3, and 24 h post-PTZ exposure. The TL was expressed as inflection ratio (Kasture et al., 2007). Inflexion ratio (IR) = (L0-L1)/(L1), (IR) = (L0-L2)/(L2), where L0 is the initial latency (s) at 0 h and L1 and L2 is the latency (s) at 3 and 24 h trial. Behavior recording from seizure activity and T-maze test were analyzed for locomotor patterns. Tracking of the locomotor pattern was done by using computer software SMART v3.0-Panlab Harvard Apparatus®.

Brain Harvesting

At the end of the behavioral studies, fish brains were harvested. The brain from each group was further divided into two sets and transferred into trizol for gene expression studies and methanol for LC-MS/MS respectively. The Brain was harvested by removing the skull of the fish and extracting the brain directly into the respective solvent. Brains were stored immediately at −80°C until further use.

Neurotransmitter Analysis Using LC-MS/MS

Important neurotransmitters like GABA, glutamate, and acetylcholine (ACh) were analyzed using LC-MS/MS. Stock solutions of 1 mg/ml were prepared for all the standard neurotransmitters in methanol (0.1% formic acid). The solution was kept at 4°C until use.

Serial dilution from 100 to 2000 ppb was used for calibration. The brain was homogenized in 200 μl ice-cold methanol (1% formic acid). The homogenate was vortex-mixed for 1 min and then centrifuged at 18,000 × *g* for 10 min at 4°C. Finally, the supernatant was pipetted and placed into vials for LC-MS/MS analysis.

LC–MS/MS was run on an Agilent 1290 Infinity UHPLC, coupled with Agilent 6410 Triple Quad LC/MS, ZORBAX Eclipse plus C18 RRHD 2.1 × 150 mm, 1.8-micron (P/N 959759-902) column, the auto-sampler system (Agilent Technologies, Santa Clara, CA, United States). The samples were separated on a SMol-RRHD-Eclipse-C18-8 (15) UHPLC-160129-00011-Pos-DMRM used at 30°C. The mobile phase consisting of 0.1% formic acid in water (Solvent A) and acetonitrile with 0.1% formic acid (Solvent B) was used with a gradient elution: 0–3 min, 50% B; 3–6 min, 95% B; 06–07 min, 95% B at a flow rate of 0.1 ml/min. ESI-MS/MS Conditions were set as follows: ESI ion source, positive ion polarity, gas temperature 325°C, drying gas flow 9.0 L/min, nebulizer pressure 45 psi, Vcap 4000V. MS acquisition of GABA, Glu, ACh as performed in electrospray positive ionization multiple reaction monitoring (MRM) mode.

Gene Expression

All brain samples were collected in ice-cold 200 μl TRIzol® reagent (Invitrogen, Carlsbad, CA, United States) and stored at −80°C until use. Gene expression study was carried out for Neuropeptide Y (NPY), Brain-derived neurotrophic factor (BDNF) and cAMP-responsive element-binding protein1 (CREB1) genes.

Isolation of RNA and First Strand cDNA Synthesis

Total mRNA were isolated by following the manufacturer's protocol. In brief, brain tissues were appropriately homogenized in TRIzol® reagent followed by mixing with chloroform and centrifuged at 13,500 rpm (revolutions per minute) for 15 min at 4°C. The upper aqueous supernatant was transferred into new tubes and isopropanol was added, mixed and were incubated for 10 min at room temperature and later centrifuged for 10 min at 13,500 rpm at 4°C. The supernatant was discarded and the pellets were subjected for rinsing with 75% ethanol. The pellets were then left for air drying between 5 and 8 min. Finally, nuclease-free water was added to each tube to dissolve the mRNA pellet. The concentration and purity of the isolated mRNA were measured by using NanoDrop Spectrophotometer. The mRNA samples were converted to cDNA with the help of Omniscript Reverse-transcription Kit (QIAGEN).

StepOne® Real-Time PCR

Gene expression for NPY, BDNF and CREB1 were computed using real-time quantitative RT-PCR (Applied Biosystems) using QuantiTect SYRB Green dye (Qiagen, Valencia, CA, United States). All the primer sets were purchased by Qiagen (npy: Dr_npy_1_SG QuantiTect Primer Assay (QT02205763), bdnf: Dr_bdnf_1_SG QuantiTect Primer Assay (QT02125326), creb:Dr_crebbpa_1_SG QuantiTect Primer Assay (QT02197503). Samples were incubated at 95°C for 2 min prior to thermal cycling (40 cycles of 95°C for 5 s and 60°C for 15 s). Relative expression of these three genes were attained by normalizing threshold cycle (Ct) values against Ct value of eef1a1b (housekeeping gene) (2 ^ [Ct eef1a1b – Ct Gene of interest]).

Statistical Analysis

All the data in the results are expressed as Mean ± Standard Errors of the Mean (SEM). Data is analyzed by Analysis of Variance (ANOVA) followed by Dunnett's tests. The *P*-value $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ is considered as

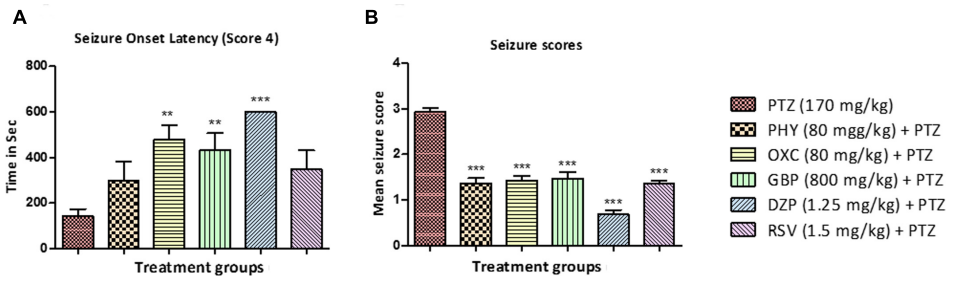

FIGURE 3 | Seizure onset latency and behavior seizure score. **(A)** Represent seizure onset latency (score 4) of AEDs treated fish when compared with PTZ treated group. **(B)** Represent the effect of AEDs on PTZ induced seizure in zebrafish. Data are expressed as Mean ± SEM, n = 10 and statistical analysis by one-way ANOVA followed by Dunnett test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

statistically significant. All the groups were compared with the PTZ negative control group.

RESULTS

Seizure Onset Latency and Seizure Score Analysis

All the animals in the PTZ treated (Group II) reached seizure score 4 within 150 – 180 s after PTZ administration. In contrast, the onset was delayed in animals treated with standard AEDs as shown in **Figure 3A**. Drugs like, OXC, GBP, and DZP significantly delayed the onset of seizure whereas delay in onset with PHY and RSV was statistically insignificant. Seizures are measured using a special scoring system (**Table 1**). All the animals treated AEDs displays seizure not more than score 2 and exhibited seizure suppression activity. Thus a significant anti-epileptic activity was observed in all the animals treated with AEDs as shown in **Figures 3A,B** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Locomotor Pattern

The locomotor pattern in the vehicle control group was demonstrated by normal swimming all over the tank. The PTZ group has spontaneously provoked seizures which is represented by abnormal and circular tracking pattern. The locomotor tracking pattern of PHY, RSV, OXC, GBP and DZP treated groups showed attenuation of PTZ seizure effect and a swimming pattern nearly similar to control group as shown in **Figure 4A**. The total distance traveled was significantly higher in all AED treated groups as compare to PTZ group. The total distance traveled was higher in control group as compare to PTZ group but it was found to be statistically insignificant as shown in **Figure 4B**. The control fish have spent the equal duration of time in both the halves of the tank, whereas PTZ group was found to be inconsistent in their swimming and spent more time in the lower half compared to the upper half of the tank. As fish were protected from seizure by treatment of AEDs, drug treatment reversed the PTZ seizure effect and time spent in lower half of the tank. So, AED treated group spent more time in upper half than compare to PTZ group as depicted in **Figures 4C,D** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Zebrafish T-maze Test for Anti-epileptic Drugs

The control group showed less to no repeated entry into the wrong arm whereas PTZ group exhibited an opposite effect, so the time spent and total distance traveled was found to be significantly less as compared to PTZ treated group as shown in **Figures 5A,B**. T-maze tracking pattern of GBP and DZP shows single wrong entry in the T-maze. Whereas PHY, RSV and OXC treated group showed repeated entry to the wrong arm. Time spent in wrong arm by DZP, OXC, GBP and RSV was found to be significantly less as compared to PTZ group. PHY showed no significant reduction in time spend in wrong arm as shown in **Figure 5B**. AEDs treated group took the high/long time to reach the deepest chamber, and time spent in the wrong arm was also increased exhibiting impaired memory functions similar to PTZ group as shown in **Figure 5C**. Fish from vehicle control group exhibited an improved IR (memory function) at 3 and 24 h in the absence of seizures. PTZ treated group exhibited decreased IR both at 3 and 24 h. All the AEDs do not significantly increase IR (memory function) at 3 and 24 h when compared with PTZ group as shown in **Figures 5D,E**. In AED-treated groups, PTZ challenge had affected their IR, none of the AEDs significantly improved memory function when compared with PTZ group ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Estimation of Neurotransmitters by LC/MS-MS

Neurotransmitter analysis showed that, as PTZ is a GABA-$_{A}$ receptor blocker the levels GABA in PTZ treated group were lower compared to control. No significant increase in GABA was found in PHY, OXC and DZP treated groups when compared to the PTZ group. GBP and RSV exhibited a significant increase in GABA as compared to the PTZ group as shown in **Figure 6A**. The level of glutamate in PTZ treated group was higher as compared to control group. Fish treated with AEDs significantly protected against PTZ induced glutamate surge and maintained glutamate levels similar to control group as shown in **Figure 6B**. Brain acetylcholine levels were significantly decreased by the PTZ group as compared with control. All the AEDs except DZP showed a similar ACh level as of PTZ group. DZP was the only

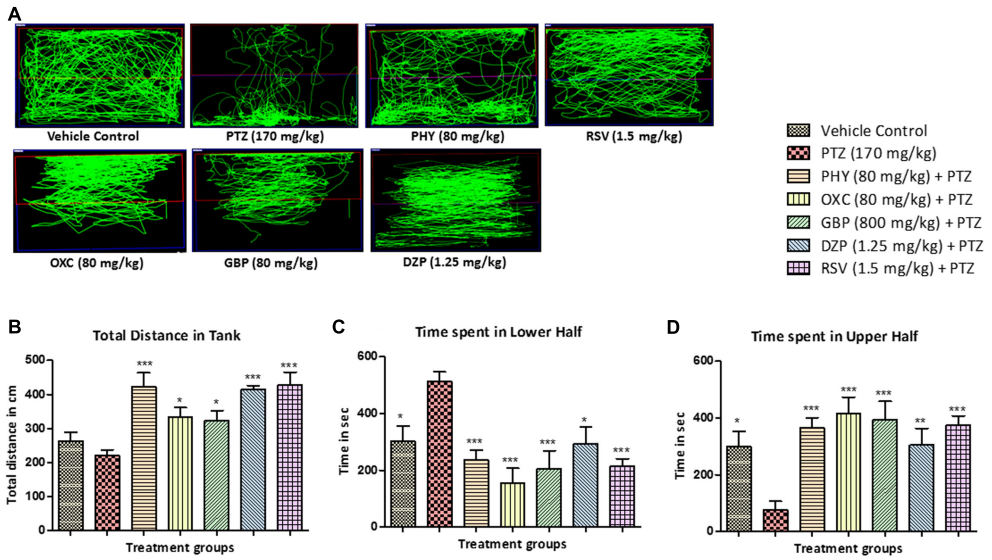

FIGURE 4 | Pentylenetetrazole (PTZ) induced locomotor pattern and behavior. **(A)** Representative locomotion tracking patterns of vehicle control, PTZ treated and all the AEDs treated groups. **(B)** Represent the total distance traveled by the fish in experiment tank in different groups. **(C,D)** Represent the total time spent by each fish in the lower and upper half of the experimental tank. Data are expressed as Mean ± SEM, $n = 10$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

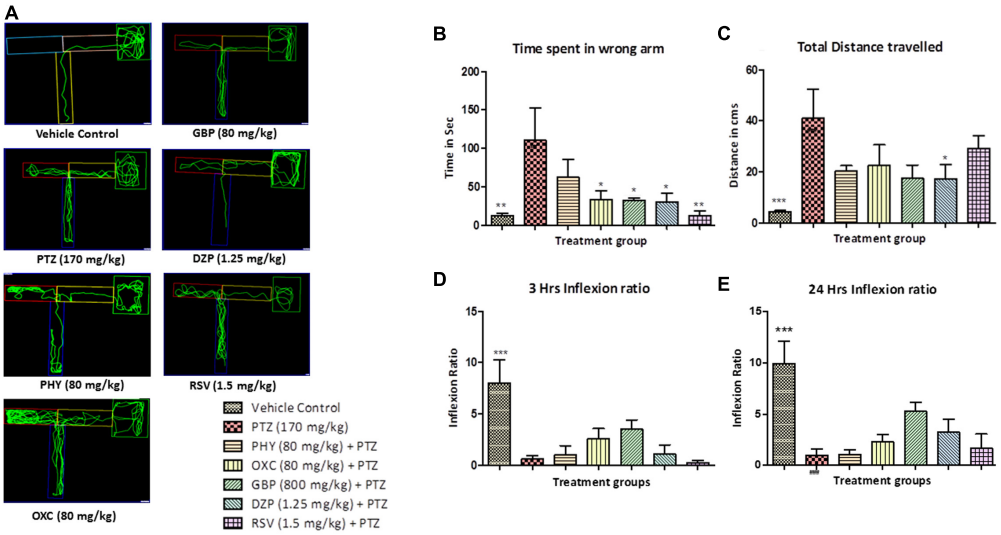

FIGURE 5 | T-maze analysis and tracking pattern. In **(A)** each plot represents locomotor pattern of vehicle control, PTZ treated and all the AEDs treated groups. **(B,C)** represent total time spent in the wrong arm of T-maze by each fish and total distance traveled to reach the deeper chamber. **(D,E)** represents graph plot of inflecion ratio at 24 and 3 h T-maze trial. Data are expressed as Mean ± SEM, $n = 10$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

drug that exhibited increased ACh levels as shown in **Figure 6C** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

Estimation of Gene Expression by RT-PCR

Brain-derived neurotrophic factor mRNA levels were downregulated in the PTZ treated group when compared with control. BDNF mRNA expression in PHY and DZP was found to be statistically insignificant. However, OXC, GBP, and RSV significantly upregulated mRNA expression of BDNF, as compared to PTZ treated group as shown in **Figure 7A**. CREB1 mRNA expression was up-regulated in the PTZ treated group as compared to the vehicle control group. In all AEDs treated fish except DZP and RSV, the mRNA expression of CREB_1 was similar as that of PTZ group, however, PHY, OXC, and GBP

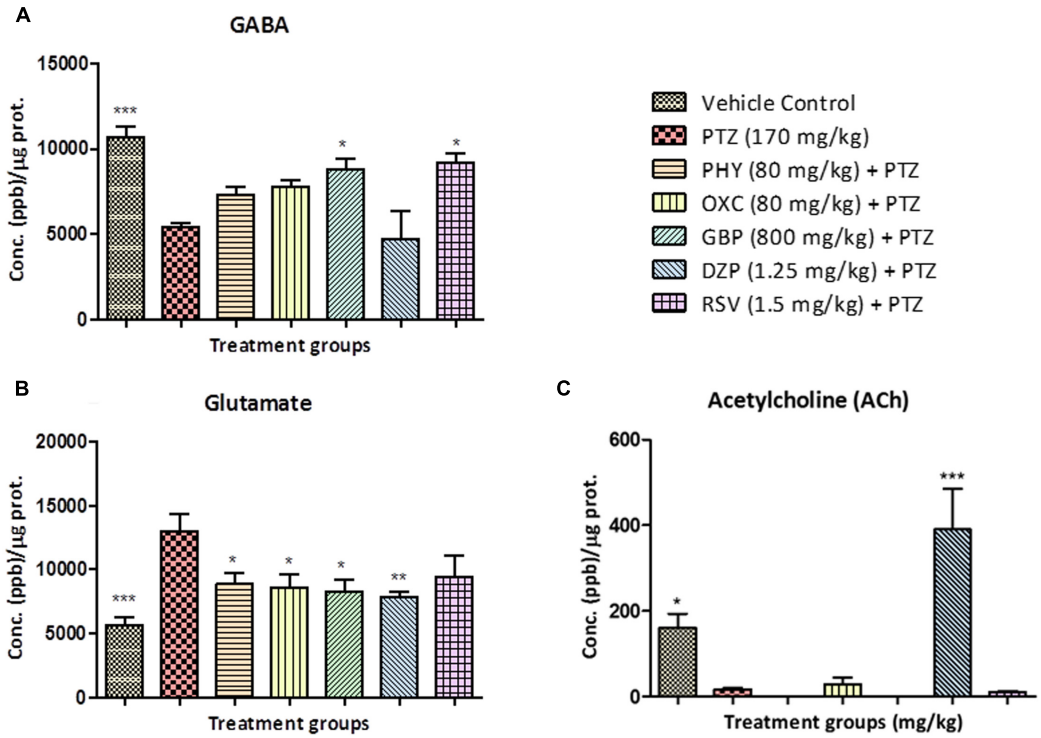

FIGURE 6 | Concentration of neurotransmitters in zebrafish brain after T-maze test. Figure represents zebrafish neurotransmitter levels. **(A)** GABA, **(B)** Glutamate **(C)** Acetylcholine. Data are expressed as Mean ± SEM, $n = 6$ and statistical analysis by one-way ANOVA followed by Dunnett test $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

significantly upregulated the expression as shown in **Figure 7B**. NPY mRNA expression was downregulated in the PTZ treated group. However, this down-regulation was ameliorated by DZP pre-treatment as compared with PTZ group. This effect was same as observed in the control group. There was no significant difference observed in PHY, OXC and RSV pre-treatment when compared with PTZ treated group as shown in **Figure 7C** ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

DISCUSSION

This work present development of a new model for epilepsy-induced cognitive dysfunction in zebrafish. This model has shown the effect of epilepsy on behavior, locomotion, important neurotransmitters and related genes expression. A considerable modulation in the parameters related to cognition such as learning and memory, proteins and genes were observed after epilepsy seizures. Effect of different AEDs' was evaluated using the developed model. Findings of the this study suggests that AEDs significantly reverse the epileptic conditions but have some negative impact on the cognitive functions of the zebrafish.

For studying various brain disorders, zebrafish (*Danio rerio*) are gaining importance and emerging as a promising model organism (Guo et al., 2015). Seizure-like behavioral responses can be induced in adult zebrafish by several pharmacological approaches and there is an increasing trend of utilizing zebrafish model in epilepsy research (Kalueff et al., 2014). They are smaller in size and easy to maintain. Furthermore, it is a diurnal species with several fundamental similarities to humans (Stewart et al., 2014). As there is a limited spectrum of antiepileptic drugs (AEDs) available. A lot of patients are resistant to the existing therapy and many suffer from drug related comorbidities. In order to develop safe and efficient AEDs, there is a lack of model systems that fully recapitulate the condition of epilepsy induced cognitive dysfunction (Phillips and Westerfield, 2014). Zebrafish as an alternative to existing animal models is gaining popularity in the field of cognitive research. There are several reports which provide strong evidence on the usefulness of zebrafish model for studying cognitive functions (Stewart and Kalueff, 2012).

Pentylenetetrazole is a chemoconvulsant act via $GABA_A$ receptor and act on an allosteric site (Desmond et al., 2012). A dose of 220 mg/kg of PTZ produce clonic tonic seizure in adult zebrafish (Banote et al., 2013). In the present study, a dose of 170 mg/kg was used as ethics committee suggested to use a lower dose to prevent further distress. Mussulini et al., 2013, utilized PTZ exposure to fish by dissolving pro-convulsant in the tank water (Mussulini et al., 2013). Whereas, IP injection in adult zebrafish was introduced by Kinkel et al. (2010). One of the method for administration of test substances to zebrafish is by dissolving the drug or chemical into the tank water, and it expected that test substance will be taken up by the fish. Nonetheless, tank water method do not give any idea about how much of the drug is actually absorbed or taken up

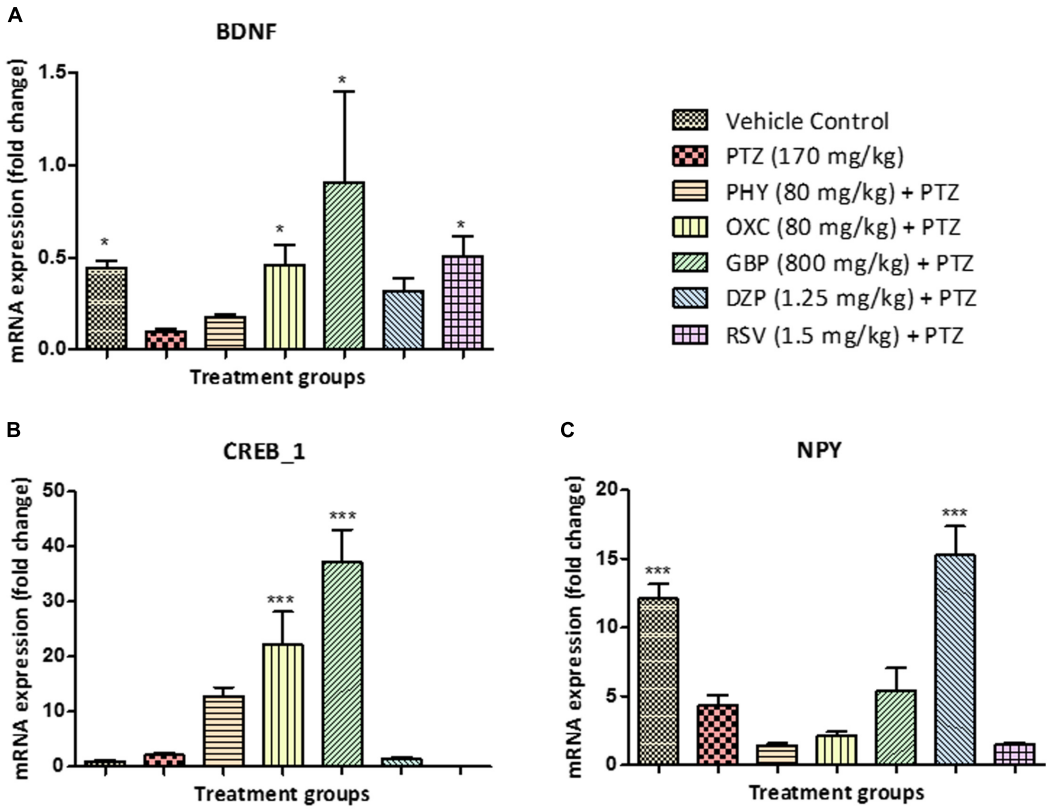

FIGURE 7 | Gene expression in zebrafish brain determined by real time-PCR. **(A)** represents graph plot for BDNF mRNA expression in zebrafish brain, the level of BDNF fold change was found to be high in AEDs treated and control group as compared to PTZ treated group. **(B)** represents graph plot of CREB_1 mRNA expression levels in zebrafish brain, the level of CREB_1 fold change were found to be high in AEDs treated as compared to PTZ treated group. **(C)** represents graph plot of NPY mRNA expression in zebrafish brain, the level of NPY fold change were found to be high in DZP treated and control group as compared to PTZ treated group. This result shows that AEDs alters the gene expression fold change in the brain and affect the cognitive functioning of the brain. Data are expressed as Mean ± SEM, $n = 6$ and $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

by the fish (Kinkel et al., 2010). Intraperitoneal injection is considered as a better option to deliver a definite amount of drug to each fish, based on body weight calculations (10 μl for 1 gm fish). This route is of greater significance in order to correlate drug concentration to the efficacy and also important in metabolic studies (Traver et al., 2003). Intraperitoneal injection is relatively safe with zero mortality when tested in our laboratory. Hence, IP route of drug administration was preferred in our research.

In the present study, we have used seizsure score as described by Mussulini et al. (2013) with minor modifications. All the AEDs tested, delayed the onset of seizure in zebrafish after PTZ insult. This indicates the positive role of AEDs in preventing the seizure episode in patients (Patsalos et al., 2008). Latency to seizure score 4 is the time required by the fish to reach seizure score 4 in a given time frame of 600 s. Drugs like PHY, OXC, GBP, DZP and RSV significantly reduced seizure score when compared to PTZ treated group. The animals in the PTZ treated (Group II, negative control) exhibited seizure-like activity and full blown seizures of score 4 as shown in **Figures 3A,B**. Seizure scores mimic the clinical condition in the patients (Huang et al., 2001). Higher the score, more intense is the seizure episode. All the AEDs significantly helped in reducing the severity of the seizures as indicated by seizure score less than 2. It is also depicted by locomotor pattern as described earlier. Locomotor pattern clearly explains the abnormal behavior in PTZ group. The behavioral analysis and locomotor tracking patterns explain that the AEDs helped fish to overcome the PTZ effect and restored usual swimming movements nearly all over the tank. As seen in PTZ treated group the locomotor moment of the fish was observed to be at the bottom most part of the tank and time spent by fish was also more, which is similar to clinical stupor like behavior and anxiety in epileptic condition.

Seizures are the uncontrolled firing of neurons which produces involuntary moment of the body, which could be a partial or generalized type of seizures (Berg et al., 2010). Since greater the seizure frequency-duration and severity, it is likely to increase cognitive impairment in patients (Inoyama and Meador, 2015). Epidemiological studies reveal that dementia or memory problems is largely a unseen problem and the number is increasing (Farooq et al., 2007). Cognition in epilepsy can adversely be affected by multiple factors, including the seizure etiology, hereditary factors, psychosocial factors, and sequelae of epilepsy treatment, including AEDs (Motamedi and Meador,

2003). AEDs, primarily affects psychomotor speed, vigilance, and attention. Secondarily, it disturbs cognitive functions like memory and learning abilities (Chung et al., 2007). Children with age below 12 have a developing nervous system which is more susceptible to the long-standing side effects of AEDs-induced cognitive impairment. It is a serious problem and it is imperative to recognize and strategies to subside the negative effect of AEDs on cognition (Lagae, 2006). In a similar way, pharmacodynamic and pharmacokinetic factors both in combination are responsible for the cognitive effects of AEDs in individuals. For example, in the clinical setting, levetiracetam (LEV) and carbamazepine (CBZ) has reported adverse pharmacodynamic interactions (Sisodiya et al., 2002). LEV, TPM, CZB and other AEDs exhibit toxicity during combination therapy having a pharmacodynamic interaction (Perucca, 2006). Switching to another drug may help in preventing the damage and in improving cognitive function, memory, alertness, or ability to concentrate. Reports also shown that shifting from multi-drug therapy to monotherapy has been advantageous in reducing cognitive adverse effects (Vining et al., 1987; Wenk, 2001).

T-maze is used as an assessment tool which provides the brief overview of the cognitive status in a zebrafish model. T-maze is one of the most widely used behavioral paradigm to study detailed features of spatial working memory (Wenk, 2001). In this repetitive learning task, individual zebrafish were trained to explore and travel to the deeper end of the T-maze. Correct choices were rewarded with big space and favorable environment and incorrect choices result in getting small congested environment confinement (Lamb et al., 2012). In the present study, AEDs have significantly ameliorated seizure provoked by PTZ exposure but they have a negative impact on cognitive functions. Results from the T-maze test clearly demonstrated that AED-treated fish gets repeatedly lost in the T-maze, which shows epilepsy-related impaired spatial memory function in zebrafish. It is also observed that zebrafish have the capabilities to successfully learn, navigate and discriminate between wrong and right arm to reach the deeper chamber. Drugs like OXC, GBP and DZP have less negative impact than PHY and RSV on memory functions. The T-maze locomotor pattern seen in PTZ group was found to be significantly different as compared to control group. Most of the AEDs exhibit a similar locomotor pattern as of PTZ treated group in T-maze. The telencephalon connected to the olfactory organ is responsible for governing spatial memory, reproductive behavior, feeding behavior, and color vision in zebrafish. The spatial memory is accountable for recording information of individuals's environment. A fish with poor working spatial memory may result in, getting repeatedly lost in the maze (Yu et al., 2006). The alteration in the neurotransmitter by AEDs and by epilepsy contributes to cognitive dysfunction in epilepsy patients (Greengard, 2001). There is a greater evidence available which explains the negative impact of AEDs on different aspects of cognitive functions.

Neurotransmitters are chemical agents in the body that modulate, initiate, and amplify signals across the brain (Thippeswamy et al., 2011). Abnormal alteration of neurotransmitters levels has been found to be closely associated with many neurological diseases including epilepsy (Huguenard, 2003). One of the reasons for the epileptic seizure and cognition in clinical conditions is neurotransmitter modulations by both epilepsy and AEDs (Park and Kwon, 2008; Kleen et al., 2010). LC–MS/MS method was used to quantify the neurotransmitters simultaneously in zebrafish brain (Santos-Fandila et al., 2015). It is well studied in rodents that neurotransmitters plays a significant role in modulating memory and learning function (Levin and Cerutti, 2009). A similar modulation was found in zebrafish brain when tested after T-maze test for learning and memory. GABA is an inhibitory transmitter in the central nervous system and known for its role in epilepsy (Shaikh et al., 2013). In the current research, all the fish treated with PTZ shows decrease level of GABA that contribute to epileptogenesis. The level of GABA was found to be high in the control group and some of the AEDs like DZP and GBP which acts via a GABAergic pathway. Drugs such as PHY and OXC which do not act via this pathway showed reduced GABA levels. A study performed by Silva (2003) in rodent, proves the fundamental role of GABA in controlling epilepsy (Silva, 2003; Werner and Covenas, 2011). The Increase in glutamate (Glu), an excitatory amino acid, is closely related to the etiology of epilepsy. This glutamate release causes a cascade of changes resulting in increased intracellular calcium that ultimately results in cell death (Holmes, 2002). It was found that level of glutamic acid in zebrafish brain of the control group was low as compared to PTZ treated group. In most of the AEDs treated group the level of glutamate was found to be high, which might have contributed to the neuronal loss, and impaired the memory function. By modulation of glutamate signaling, many biological events are related to brain functioning are affected (Ozawa et al., 1998), such as learning and memory (Izquierdo and Medina, 1997). Acetylcholine (ACh), a signaling molecule elicit several actions at neuromuscular junctions and in the CNS and also plays a key role in modulating glutamate release and maintaining memory formation. (Atzori et al., 2003). It was found that level of ACh in PTZ and all the AEDs treated group was low as compared to control group and this is in correlation with high levels of glutamate. Memory function was not improved due to the low level of ACh release in most of the AEDs treated group. But ACh was found to be high in DZP group and which could be due to acetylcholinesterase (AChe) inhibition property of DZP (Lundgren et al., 1987). Although the level of ACh in DZP treated group was high, there was not much improvement in memory functions in zebrafish when tested in T-maze.

Brain-derived neurotrophic factor, a member of the "neurotrophin" family, enhances the survival and differentiation of several classes of neurons (Mercer et al., 1997). In our result, we found that level of BDNF mRNA expression (fold change) in PTZ treated group was decreased. Similarly, we found that there was no significant difference in BDNF mRNA expression (fold change) in PHY and DZP treated groups, which showed that AEDs contributes toward memory impairment although correcting epilepsy. Fish treated with OXC, GBP, and RSV showed a slight increase in mRNA expression of BDNF gene. A study conducted by Binder (2004), have shown that

upregulation of BDNF is involved in epileptogenesis, and modulation of BDNF signal transduction helps in preventing an epileptic condition (Binder, 2004). The transcription factor, CREB is crucial for important functions of cognition like memory and synaptic plasticity. Earlier studies reported an increase in CREB phosphorylation in rodent models of epilepsy (Zhu et al., 2012). In all the fish treated with AEDs, the level of CREB mRNA expression (fold change) was higher than PTZ treated group. This states that AEDs along with epilepsy contributes to loss of memory function in zebrafish after 24 h T-maze trial. The level of CREB mRNA expression was high in PTZ treated fish which shows that CREB too plays the important role in the process of epileptogenesis. Theories around cAMP-response element binding protein (CREB) and memory are still developing. Formation of long lasting memory is completely dependent on activation of (CREB)-dependent gene expression which is a crucial phase in the molecular pathway. Lower the expression of CREB gene higher will be the memory function (Benito and Barco, 2010). NPY play a significant role in regulating various physiological events in the brain, including energy balance, learning and memory, and epilepsy. NPY gene is generally responsible for regulation of neurotransmitters in the brain. It is reported that NPY gene therapy decreases chronic spontaneous seizures in a rat model of temporal lobe epilepsy (Noe et al., 2008). It selectively reduced synaptic excitation mediated by glutamate release (Hollopeter et al., 1998). In the zebrafish brain, neurons containing NPY mRNA are widely distributed in particular to regions like telencephalon, optic tectum, and rhombencephalon (Soderberg et al., 2000). In our result we found that level of NPY mRNA expression (fold change) in PTZ treated group was decreased. The mRNA expression of NPY gene in control group was found to be high. Similarly, fish treated with DZP and GBP, showed an increase in the mRNA expression of NPY gene. We found that there was no significant difference in NPY mRNA expression (fold change) in PHY, OXC, and RSV-treated group as compared with PTZ group. A study performed by Stroud et al., demonstrated that NPY suppresses absence seizures in Genetic Absence Epilepsy Rats of Strasbourg (GAERS) (Stroud et al., 2005). NPY also play a significant role in human epilepsy and it is supported by an increased NPY expression in biopsy samples from temporal lobe epilepsy patients (Furtinger et al., 2001).

This study suggest that the negative impact of standard anti-epileptic drugs on learning and memory, which is very common among epileptic patients. The successful development of zebrafish model was confirmed by the effect of epilepsy and AEDs impairing the learning and memory abilities in zebrafish. It was found that AEDs suppresses seizure-like behavior but cannot reverse seizure associated learning and memory dysfunction in a zebrafish model. This model, therefore, potentially extend the significant use of zebrafish and technique for screening or developing newer drugs for epilepsy-related cognition dysfunction in humans.

CONCLUSION

For investigating the cause and pathology of human disease animal models are considered as a useful tool. It is better known that such models can never represent the complete pathology that is observed in human diseases. To develop a model in the animal for brain disorder particularly epilepsy is very difficult because of its disease complexity and variation among the species. However, by using a number of different techniques and parameters in the zebrafish, we can incorporate the unique feature of specific disorder to study the molecular and behavior basis of this disease. The behavioral study, neurotransmitter analysis and gene expression studies in the aforementioned work above provide a first proof-of-principle model for screening basic drugs in epilepsy-related cognitive research using zebrafish as a choice of animal.

ETHICS STATEMENT

The experimental protocol was approved by the Monash Animal Research Platform (MARP) Animal Ethics Committee, Monash University, Australia (MARP/2016/009).

AUTHOR CONTRIBUTIONS

UK performed all the experimental procedure along with result analysis, manuscript writing and figures designing. YK contributed in designing gene expression study, result analysis and figures in the manuscript. IO contributed in LC-MS/MS method. MS contributed in designing the study, result analysis and manuscript writing.

FUNDING

This research work was supported by the eScience Fund of Ministry of Science, Technology and Innovation (MOSTI), Malaysia (Grant No. 06-02-10-SF0250).

ACKNOWLEDGMENT

The authors acknowledge Ms. Rufi Tambe for helping with proof reading and language editing of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 15 August 2017
doi: 10.3389/fphar.2017.00536

Rewarding Effects of Operant Dry-Licking Behavior on Neuronal Firing in the Nucleus Accumbens Core

Enrico Patrono, Jumpei Matsumoto, Hiroshi Nishimaru, Yusaku Takamura, Ikhruud C. Chinzorig, Taketoshi Ono and Hisao Nishijo*

System Emotional Science, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan

OPEN ACCESS

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***Correspondence:**
Enrico Patrono
e.patrono@gmail.com

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *12 June 2017*
Accepted: *02 August 2017*
Published: *15 August 2017*

Citation:
Patrono E, Matsumoto J, Nishimaru H, Takamura Y, Chinzorig IC, Ono T and Nishijo H (2017) Rewarding Effects of Operant Dry-Licking Behavior on Neuronal Firing in the Nucleus Accumbens Core. Front. Pharmacol. 8:536. doi: 10.3389/fphar.2017.00536

Certain eating behaviors are characterized by a trend of elevated food consumption. However, neural mechanisms mediating the motivation for food consumption are not fully understood. Food impacts the brain-rewarding-system via both oral-sensory and post-ingestive information. Recent studies have reported an important role of visceral gut information in mediating dopamine (DA) release in the brain rewarding system. This is independent of oral sensation, suggesting a role of the gut-brain-DA-axis in feeding behavior. In this study, we investigated the effects of intra-gastric (IG) self-administration of glucose on neuronal firings in the nucleus accumbens (NA) of water-deprived rats. Rats were trained in an operant-licking paradigm. During training, when the light was on for 2 min (light-period), rats were required to lick a spout to acquire the water oral-intake learning, and either an IG self-infusion of 0.4 M glucose (GLU group) or water (H_2O group). Rats rested in the dark-period (3 min) following the light-period. Four cycles of the operant-licking paradigm consisting of the light–dark periods were performed per day, for 4 consecutive days. In the test session, the same rats licked the same spout to acquire the IG self-administration of the corresponding solutions, without oral water ingestion (dry licking). Behavioral results indicated IG self-administration of glucose elicits more dry-licking behavior than that of water. Neurophysiological results indicated in the dark period, coefficient of variance (CV) measuring the inter-spike interval variability of putative medial spiny neurons (pMSNs) in the NA was reduced in the H_2O group compared to the GLU group, while there was no significant difference in physical behaviors in the dark period between the two groups. Since previous studies reported that DA release increases CV of MSNs, the present results suggest that greater CV of pMSNs in the GLU group reflects greater DA release in the NA and elevated motivation in the GLU group, which might increase lickings in the test session in the GLU group compared to the H_2O group.

Keywords: intra-gastric self-administration, glucose, operant dry-licking, nucleus accumbens, single unit recording

INTRODUCTION

Eating behaviors are occasionally characterized by a trend of elevated food consumption. Such behavior leads to the prevalence of eating disorders such as obesity and binge eating disorder (BED). Recent studies suggest that BED shows specific features involving uncontrolled compulsive food consumption and feelings of loss of control over eating behavior (American Psychiatric Association, 2013), underlined by neurobiological features of dysfunctional cognitive control, food addiction, and gene–environment interactions posing as risk factors (Latagliata et al., 2010; Patrono et al., 2015, 2016; Duarte et al., 2014, 2015). Studies have shown that a low availability of dopamine D2 receptors (DA D2Rs) in the nucleus accumbens (NA) is a genetic risk factor for chocolate compulsive-seeking behavior, which is also mediated by stressful environments (Hoebel et al., 2009; Campbell et al., 2010; Kenny, 2011; Di Segni et al., 2014). This supports the idea that a complex gene–environment interaction plays a key role in the development of maladaptive compulsive eating behavior (Campbell et al., 2010; Patrono et al., 2015).

Food exerts its reinforcing effects on the brain reward system via both gustatory (oral-sensory) and post-ingestive pathways (Li et al., 2002; Jang et al., 2007; Margolskee et al., 2007; Dotson et al., 2010; Fernstrom et al., 2012). A previous neurophysiological study reported that intragastric (IG) infusion of amino acids changed neuronal activity in the lateral hypothalamus and amygdala (Davaasuren et al., 2015). Furthermore, studies have suggested that hepato-portal glucose sensors, which act as an unconditioned stimulus for the acquisition of a learned-food-preference (Delaere et al., 2013) and regulates several physiological functions such as glucose utilization (Burcelin et al., 2000), may directly influence dopaminergic activity. A functional magnetic resonance imaging (fMRI) study using rats reported that IG infusion of glucose activated the NA in less than 10 min (Tsurugizawa and Uneyama, 2014). This suggests a potential role for autonomic afferents innervating the hepato-portal system, in peripheral-glucose-sensing and communication with brain-reward-circuits (Delaere et al., 2013). However, recent evidences have demonstrated an important role for afferent information from the gut in mediating DA release in the reward-system, which stimulates food intake without depending on oral sensation (de Araujo et al., 2008; Ren et al., 2010; Sclafani et al., 2011; Tellez et al., 2013). Thus, nutrient-related DA efflux is induced directly by gastrointestinal tract stimulation, suggesting a gut-brain-DA-axis involved in feeding behavior (de Araujo et al., 2012).

Behavioral studies have reported that positive signals (e.g., glucose intake) serving as unconditioned stimuli (US) in flavor-preference learning tasks are generated in the intestine, and that post-absorptive glucose could condition food and place preferences in rats (Ackroff et al., 2010; Oliveira-Maia et al., 2011; Zukerman et al., 2013). Specifically, the conditioning procedure increased the intake of the flavored conditioned stimulus. In this procedure, oral-intake of flavored conditioned stimuli (CS) is accompanied by IG self-infusion of glucose (US), which suggests that the rewarding effects of glucose are mediated by post-oral processes (Ackroff and Sclafani, 2014, 2015; Sclafani and Ackroff, 2016). These findings suggest that post-ingestive nutrient-conditioned preference and subsequent intake-stimulation are mediated by an "appetition" system, which is different from an intake-suppressor "satiation" system (Sclafani, 2013). Recently, a new conditioning paradigm has been introduced to investigate the post-ingestive nutrient-control-of-food reward (de Araujo et al., 2008; Sclafani et al., 2015). In this paradigm, subjects are trained to lick a sipper spout to receive IG nutrient infusions. During the test sessions, the spout is empty (i.e., without any solution), which allows a direct probe of sensitivity to post-oral detection of infused nutrients. The operant dry-licking paradigm exploits spout licking (CS)-IG self-infusion of a nutrient (US) combination without feedback from oral sensation.

The aim of the present study was to investigate whether IG self-administration of glucose in deprived rats, through dry licking, affected NA neuronal firings in association with dopamine release. Thus, we recorded NA neuronal activity while animals exhibited the operant dry-licking behavioral paradigm. The role of the dopaminergic mesolimbic system in oral and post-oral nutrient conditioning has been extensively investigated (de Araujo et al., 2008; Ren et al., 2010; Sclafani et al., 2011; Tellez et al., 2013, 2016). However, to our knowledge, no previous studies have investigated single-unit neural activity in NA during IG self-administration using the operant dry-licking paradigm.

MATERIALS AND METHODS

Animals

Adult, male Wistar rats ($n = 14$, 250–350 g, SLC, Japan) were used for this study, and equally divided into two groups: glucose group (GLU, $n = 7$) and water group (H_2O, $n = 7$). GLU received IG self-infusion of glucose, while H_2O received IG self-infusion of tap water. Housing temperature was maintained at $23 \pm 1°C$, with a 12-h light/dark cycle (lights on at 07:00). Prior to surgery, two male rats were housed per cage. After the surgery, rats were individually housed, with food and water available ad libitum. All rats were treated in strict compliance with the United States Public Health Service Policy on Human Care and Use of Laboratory Animals, National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Guidelines for the Care and Use of Laboratory Animals at the University of Toyama. All experimental procedures were approved by our institutional committee for experimental animal ethics. Every attempt was made to minimize the number of experimental animals and their suffering.

Surgery

Surgical procedures have been described previously (Matsumoto et al., 2012; Davaasuren et al., 2015). Briefly, rats were anesthetized with sodium pentobarbital (40 mg/kg; intraperitoneal, i.p.). Electrode assemblies were implanted bilaterally into NA core (AP = +1.5, ML = ±1.3, DV = +6.5), according to the atlas of Paxinos and Watson (2006). The recording electrode assembly comprised four tetrodes, each of which included four tungsten microwires (20 μm in diameter; California Fine Wire), encased in a stainless steel cannula (30

gauge; Hakko, Japan), and a microdrive. The tip impedance was approximately 200 kΩ at 1 kHz. For intra-gastric cannulation, a midline incision was made in the abdominal wall. One end of a silicon tube was inserted into the gastric fundus and ligated with a silk thread. The other end of the silicon tube was passed from the abdomen under the back skin and held on the skull (Tsurugizawa et al., 2008). After the surgery, all rats were allowed to recover for 1 week and administered intra-muscular antibiotic (orbifloxacin 5%, 0.3 mg/kg; DS Pharma Animal Health, Japan). During the recovery period, the animals were monitored for signs of pain, distress, or morbidity every 12 h. When any of these signs were detected, the animals were immediately sacrificed with an i.p. overdose of sodium pentobarbital.

Apparatus

A skinner box (30 cm × 25 cm × 35 cm) was used for behavioral testing (**Figure 1A**): left/right side walls were made of stainless steel, and front/back walls were made of transparent plexiglass allowing video tracking of the animals. The right wall was equipped with two halogen lights (5 watt) and a sipper spout that protruded into the cage by pressing a lever placed just below. On the external side of the right wall, a 20 mL syringe with tap water was connected to the sipper spout. Outside the cage, an automated control-system was set up to control the operant task (turning lights on/off, spout protrusion, spout-licking detection, and delivery of solutions through the spout and IG catheter). Licks on the spout were detected by a touch sensor connected to the spout, and were counted by a computer. Moreover, an injection pump with a 20 mL syringe for IG-infusions was connected to the control-system. The automated control-system was connected to a computer (Interface GPC-2000, Interface, Japan) and ran through a Visual C++ configuration (Microsoft, Corp., United States).

Behavioral Procedures

All the behavioral procedures were conducted in a sound- and light-attenuated experimental room, during the light phase (4:00–7:00 pm). Following the surgery, physiological saline (1 mL) was flushed through the IG cannula to clean its insides, 1–3 h before behavioral procedures on all experimental days. After 1 week of post-surgery recovery, the animals were trained for several days to form an operant lights-on lever-pressing and sipper-spout association (habituation period). During the habituation period, rats could acquire water (10 μL/lick) from the spout if the rats pressed the lever when the light was turned on. After 1 day of the post-habituation period, the animals received several incidental IG-infusions of 0.4 M glucose solution (5 mL, 1 mL/min/kg) in a glucose novelty test (GNT), to avoid any novelty effect due to IG glucose infusion. After 2 days of the post-GNT period, behavioral testing began. It consisted of training (4 days) and test (4 days) sessions separated by a rest day. Before the training session, the animals were randomly divided into two groups (GLU and H_2O). Each training day consisted of one session of 20 min. Each session was comprised of four cycles of an operant task. Each cycle included two periods: (1) light and (2) dark periods. In the light period, light was turned on for 2 min, and the animals were allowed to press the lever in order to introduce the sipper spout into the apparatus, to freely lick water, and simultaneously receive IG-infusions. Each lick simultaneously delivered 10 μL of water from the spout and 10 μL of IG-infusion. The solution for IG-infusion was 0.4 M glucose for the GLU group, and tap water for the H_2O group. In the dark period, light was turned off for 3 min and the animals rested. Since it has been reported that IG infusion of glucose increased blood-oxygen-level dependent (BOLD) signals in NA in less than 10 min (Tsurugizawa and Uneyama, 2014), and because BOLD signals have a poor temporal resolution, we considered that a 5-min-cycle could be a useful time window for IG glucose to affect neuronal firings in NA. In the test session, the same protocols as those in the training session were used, except that the sipper spout was dry (i.e., without water). The

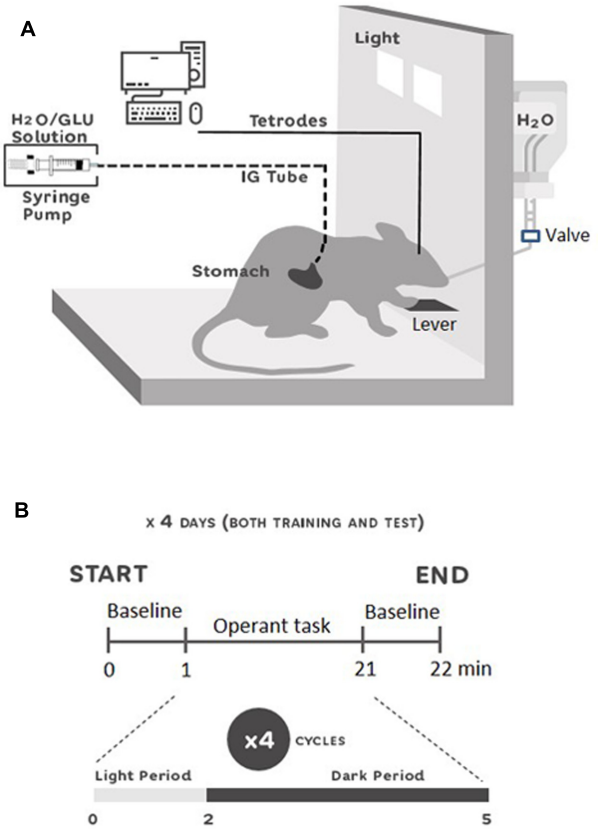

FIGURE 1 | Experimental setup **(A)** and single unit recording schedule **(B)**. **(A)** Skinner box used for the experiments equipped with a flat plastic floor, a stainless steel lever, a sipper spout protruding into the cage, and two halogen lights. IG-solutions were delivered through a gastric cannula using a syringe pump. Neural activity was recorded through a cable connected to the rat's head. The rat could freely move in the chamber. **(B)** Recording schedule during the 4 days of training and test period. At 0 min the recordings were started with 1 min for baseline recording. Then, the protocol for the operant task in four cycles of 5 min started. At 21 min, the last baseline activity was recorded for 1 min. Each cycle was comprised of 2 min of light on (Light-Period), and 3 min of light off (Dark-Period). In the Light-Period, the animals of both groups had access to a spout with oral-intake of water and IG infusions in the training session, and access to a spout only with IG infusions in the test session. In the Dark-Period, animals rested.

animals were water-deprived for 20 h during all the procedures, except for the rest days during which they returned to drink water ad libitum. **Figure 1B** shows the timelines of the behavioral procedures.

Recording Procedure

A cable was connected to the socket on the rat's head, which was connected to the electrodes on the subject rat's head, and neuronal activity was recorded. The analog signal was sent to a set of amplifiers (Omniplex, Plexon, United States) and then to an analog-to-digital system (Omniplex, Plexon, United States). Neuronal activities were digitized at a 40-kHz sampling-rate. Any 0.8-ms waveforms that crossed an experimenter-defined threshold were stored for offline spike sorting via OmniPlex (Plexon, Inc., Dallas, TX, United States). Moreover, a digital camera was connected to the system, in order to track animal behavior. If no signal was found, the electrode assembly was lowered by approximately 80–100 μm, and checked again on the following day. If stable neuronal signals were identified over a 10-min period, the electrode assembly was fixed.

Neuronal activity was recorded only in training and test sessions. At the beginning of the session, animals were connected to the cable and placed into the apparatus. After a 10-min period for stabilizing the neuronal signals, the session started with 1 min baseline, a 20 min session (four cycles of the operant task), and a 1 min baseline (**Figure 1B**). When the session was completed, the rat was returned to its own cage. Neuronal activity was recorded from the same electrode location, throughout the training and test sessions.

Data Analyses

Behavioral Analysis

Behavioral data in the light/dark periods were analyzed in two different ways, across training and test sessions. In the light period, the operant dry-licking behavior was analyzed in both training and test sessions, in order to evaluate if the deprived rat was able to self-infuse IG glucose despite the absence of the oral-intake of water. In the dark period, three behavioral features (exploration, grooming, and immobility) were analyzed in the test session, to assess physical behavioral activity.

In the light period, to test the hypothesis that deprived rats are able to self-infuse IG 0.4 M glucose despite the absence of the oral-intake of rewarding solution (water), two behavioral parameters were measured: (1) total number of licks/IG-infusions in the GLU and H_2O groups in the training and test sessions; (2) lick-tendency across the 4 days in training and test sessions in GLU and H_2O groups. Total numbers of licks/IG-infusions were compared by repeated measures two-way (RMT-) ANOVA with two factors: group (GLU vs. H_2O) and task condition (training vs. test). In lick tendency, averaged daily licks in the training or test session were compared by RMT-ANOVA with two factors: group (GLU vs. H_2O) and day (days 1–4).

In the dark period, to check a possibility of non-specific correlation between NA neuronal activity and physical behavioral activity, three main behavioral features (exploration, grooming, immobility) were compared between GLU and H_2O groups in the test session. Exploratory behavior included any locomotor activity around the chamber; including rearing, sniffing, and approaching the sipper spout hole location. Grooming behavior was defined as any self-care behavior, and immobility was defined as any resting behavior. The duration (%) and frequency (per min) of the three behaviors were measured using Observer 5.0 (Noldus, The Netherlands). Behavioral data in the two groups were compared using the unpaired *t*-test, since the three behaviors are not independent.

Statistical analysis was conducted with MatLab R2015b (Mathworks, Inc., United States), or Microsoft Excel 2010 (Microsoft, Corp., United States). *p*-values less than 0.05 were considered statistically significant.

Neurophysiological Analyses

The recorded waveforms were projected to a principle component subspace using NDManager (Hazan et al., 2006[1]) and semi-automatically sorted into single neurons using KlustaKwik (Harris et al., 2000[2]), and Kluster (Hazan et al., 2006[1]) respectively, according to previous studies (e.g., Maingret et al., 2016).

It is reported that distinct types of striatal neurons differently respond to reward (Berke, 2008; Lansink et al., 2010; Matsumoto et al., 2012), and show different spontaneous firing patterns (Berke et al., 2004; Berke, 2008; Schmitzer-Torbert and Redish, 2008; Gage et al., 2010; Lansink et al., 2010; Matsumoto et al., 2012). To separately analyze the different types of neurons, they were classified based on the following three electrophysiological properties according to previous studies (Berke, 2008; Schmitzer-Torbert and Redish, 2008; Gage et al., 2010; Matsumoto et al., 2012): (1) post-spike suppression (Schmitzer-Torbert and Redish, 2008), the period that passed before neuronal activity returned to its average firing rate after each action potential; (2) spike width (peak-to-valley duration of the waveform; Gage et al., 2010); (3) mean firing rate during baseline phase. According to the previous reports (see above), putative medial spiny neurons (pMSNs) were defined as such if the spike widths were >0.37 ms, and the post-spike suppression was <50 ms. Putative fast spiking interneurons (pFSIs) were defined as such if the mean firing rates were >2 Hz, the spike widths were <0.33 ms, and the post-spike suppression was <50 ms. The neurons that did not match any of the criteria above were defined as unclassified neurons.

A previous study reported that IG-infusion induced changes in motivation and/or post-ingestive effects (Tsurugizawa and Uneyama, 2014). We hypothesized that changes in motivation and/or post-ingestive effects might be mediated by firing patterns of NA neurons in the dark period. To analyze firing patterns of NA neurons, mean firing rates and coefficient of variance (CV) (SD/mean) of inter-spike intervals in the dark period (Stern et al., 1997) were calculated and compared between the two groups using two-way ANOVA with two factors: two groups (GLU vs. H_2O) $\times$ 2 task conditions (training vs. test).

[1]http://neurosuite.sourceforge.net/

[2]http://klustakwik.sourceforge.net/

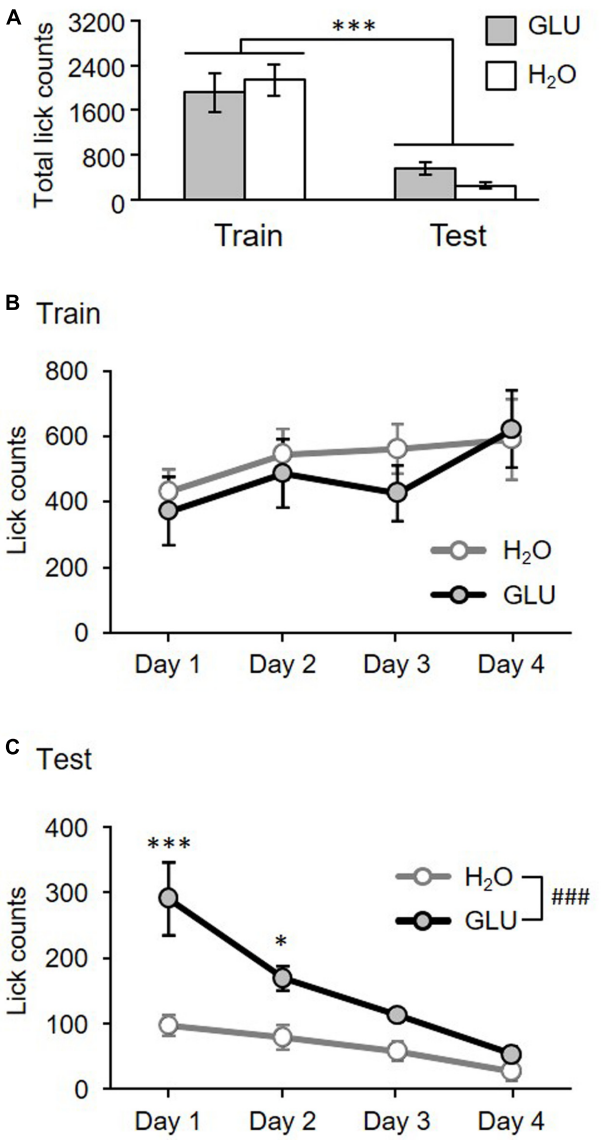

FIGURE 2 | Total number of licks for 4 days **(A)** and lick-tendency across 4 days **(B,C)** in the training and test sessions for GLU and H_2O groups. **(A)** Total number of licks for 4 days. The histograms show the total lick counts for 4 days in H_2O (white bars) and GLU group (gray bars), in Training (Train) and Test (Test) sessions. There was a significant main effect of task condition (Training vs. Test). ***$p = 4.5 \times 10^{-6}$. **(B,C)** Lick-tendency in training **(B)** and test **(C)**. Ordinates indicate averaged daily in each experimental day. **(B)** No significant differences have been found. **(C)** There were significant main effects of group, and day, and significant interaction between group and day. ###$p = 0.0017$; ***$p = 8.0 \times 10^{-7}$; *$p = 0.011$. Error bars represent ± SEM.

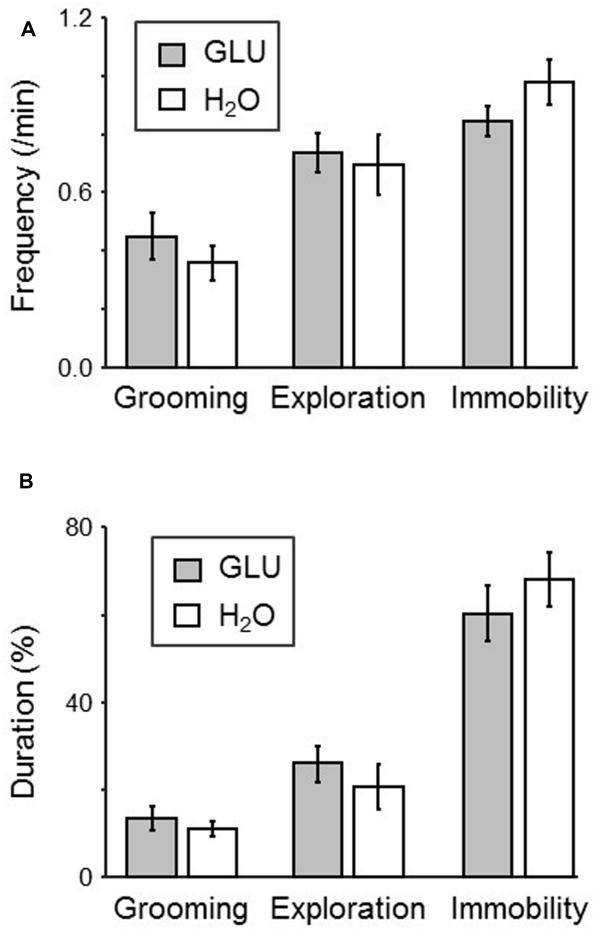

FIGURE 3 | Comparison of behaviors in the dark-period of the test session between H_2O and GLU groups. **(A,B)** Frequency **(A)** and duration **(B)** of each behavior (grooming, exploration, and immobility). No significant difference between the groups was found.

RESULTS

Operant Dry-Licking and Lick-Tendency

Figure 2A shows the total number of licks/IG-infusions in the GLU and H_2O groups during the training and test sessions. A statistical comparison by RMT-ANOVA indicated a significant main effect of task condition (training vs. test) ($F[1,12] = 61.70$, $p = 4.5 \times 10^{-6}$). There was no significant main effect of group (GLU vs. H_2O) ($F[1,12] = 0.09$, $p = 0.76$), or an interaction between the group and task condition ($F[1,12] = 2.09$, $p = 0.17$).

Figure 2B shows lick-tendency across 4 days in the training session. A statistical comparison by RMT-ANOVA indicated that there was no significant main effect of group ($F[1,12] = 0.26$, $p = 0.62$) nor interaction between group and day ($F[3,36] = 0.49$, $p = 0.69$). The results indicated that there was no significant difference between GLU and H_2O groups during the training session. However, there was a significant main effect of day ($F[3,36] = 2.98$, $p = 0.044$) indicating that lick-counts gradually increased throughout training.

Figure 2C shows lick tendency across 4 days in the test session. A statistical comparison by RMT-ANOVA indicated that there were significant main effects of group ($F[1,12] = 16.11$, $p = 0.0017$) and day ($F[3,36] = 6.17$, $p = 0.0017$), and a significant interaction between group and day ($F[3,36] = 19.62$, $p = 1.0 \times 10^{-7}$). The *post hoc* test comparisons revealed that lick-counts in days 1 and 2 were significantly larger in the GLU

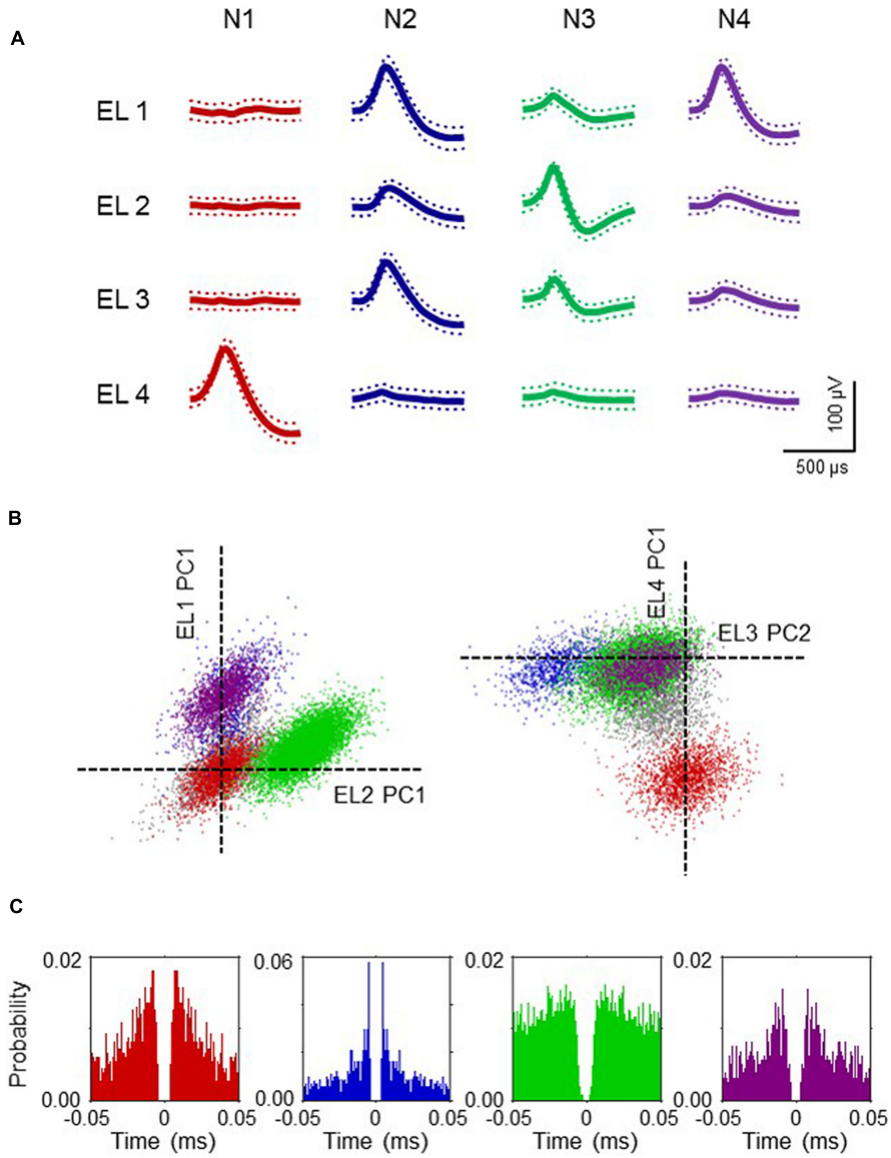

FIGURE 4 | Example waveforms of four NA neurons isolated by an offline cluster analysis. **(A)** Waveforms (mean ± SD, dotted) recorded from four electrodes (tetrode) (EL 1–4). The waveforms indicated by N 1–4 correspond to four neurons, identified by the offline cluster analysis in **(B)**. **(B)** The results of an offline cluster analysis. Each axis represents a principle component (PC1 or 2). Each dot represents one neuronal spike. The semi-automatic spike sorting resulted in the four colored clusters of neural signals. Gray dots represent unsorted spikes. The axes represent the first principle component of each of the four electrodes. Four colored clusters (red, blue, green and purple, corresponding units N 1–4 in **A**, respectively) are recognized. **(C)** Autocorrelograms of the neurons. Bin width = 1 ms. Ordinates indicate probability, where bin counts were divided by the number of spikes in the spike train.

group than the H_2O group (day 1, $p = 8.0 \times 10^{-7}$; day 2, $p = 0.011$; simple main effect test). The results indicate that overall, the GLU group licked the spout more without oral intake than the H_2O group, although licking gradually decreased across 4 days in both groups.

Behaviors in the Dark Period

Figure 3 shows comparisons of each behavior (exploration, grooming, and immobility) in the dark period of the test session between groups. The results indicated that there was no significant difference in the frequency of each behavior between groups ($p > 0.05$, unpaired t-test) (**Figure 3A**). Furthermore, the results also indicated that there was no significant difference in the duration of each behavior between groups ($p > 0.05$, unpaired t-test) (**Figure 3B**).

Neuronal Firing Patterns

The firing patterns of 191 neurons were recorded from NA. Typical waveforms of four NA neurons (N 1–4) simultaneously recorded from four wires (EL 1–4) in the same tetrode are

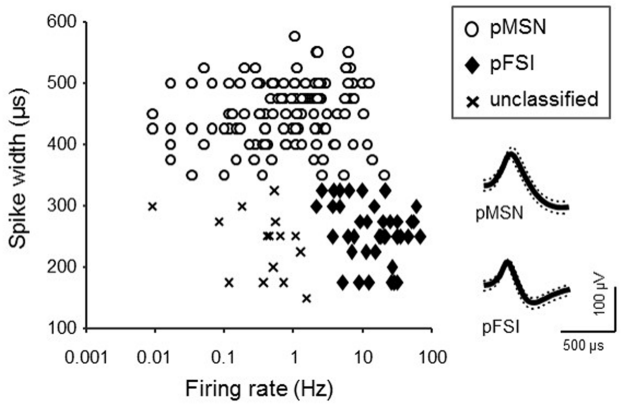

FIGURE 5 | Classification of NA neurons. The graph indicates a scatter plot showing electrophysiological properties (spike width and baseline firing rate) of each NA neuron. Symbols represent classes of NA neurons. Inset shows representative waveforms (mean ± SD, dotted lines) of pMSN and pFSI.

shown in **Figure 4A**. **Figure 4B** displays the results of spike-sorting by offline cluster cutting of the neuronal activities shown in **Figure 4A**. Each dot represents one spike, and four clusters of dots indicated by different colors were recognized. Autocorrelograms of these neurons indicated that their refractory periods were more than 3 ms, which demonstrates that these spikes were recorded from single neurons (**Figure 4C**). **Figure 5** shows three types of NA neurons based on neurophysiological parameters. A scatter plot of NA neurons, based on the neurophysiological criteria of mean firing rates and spike widths, indicated that clusters of pMSNs (open circles) and pFSIs (closed diamonds) were clearly identified. **Table 1** shows numbers of each type of NA neurons recorded in each period, per group. In the following analyses, we focused on pMSNs, because the numbers of neurons recorded in other classes were not enough to compare neural activity between groups.

TABLE 1 | Number of neurons recorded in this study.

	Glucose group		H_2O group		
	Training	Test	Training	Test	Total
pMSN	27	23	52	20	122
pFSI	14	4	12	12	42
Unclassified	9	1	10	7	27
Total	50	28	74	39	191

pMSN, putative medial spiny neurons; pFSI, putative fast spiking interneurons.

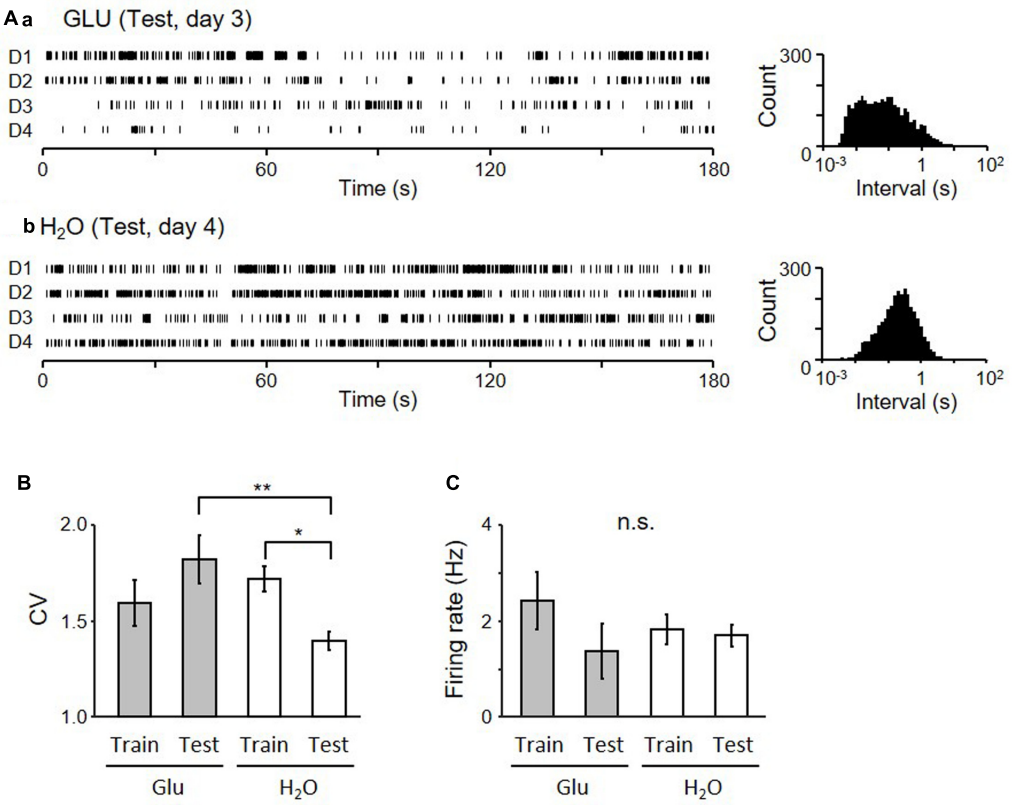

FIGURE 6 | Firing patterns of pMSNs in the dark period of the Test session. **(A)** Raster plots of neuronal activity of two neurons (a from GLU-group and b from H_2O-group) in the dark-period of four cycles (D1–4) (left) and inter-spike interval histograms of the given neurons (right). **(B)** Comparison of coefficient of variance (CV) of the inter-spike intervals of all neurons recorded during the 4 days of each session in each group. $^*p < 0.05$, $^{**}p < 0.01$. **(C)** Comparison of mean firing rates.

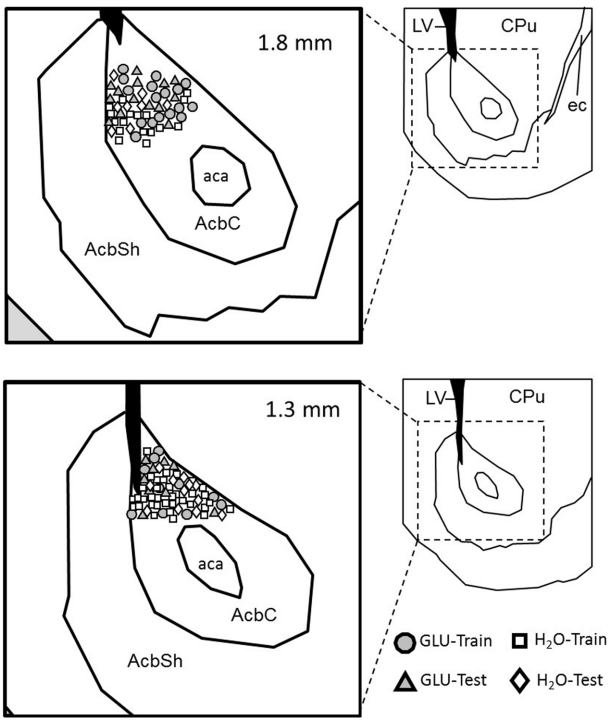

FIGURE 7 | Locations of recorded pMSNs. Estimated positions of the recorded pMSNs in each condition (Training or Test) in each group (GLU and H_2O) in coronal sections. Each inset in the right shows a low-magnified view of the corresponding section. The value on each section indicates the distance from the bregma. AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; aca, anterior commissure anterior part; CPu, caudate putamen; ec, external capsule.

Examples of firing patterns of two pMSNs in the dark period are shown in **Figure 6**. The inter-spike intervals of a neuron shown in **Figure 6A** recorded from the GLU-group are more variable than those of a neuron shown in **Figure 6B** recorded from the H_2O group, during the Test. **Figure 6B** shows a statistical comparison of coefficient of variances (CV) of the inter-spike intervals by two-way-ANOVA. The results indicated that there was a significant interaction between group (GLU vs. H_2O) and task condition (training vs. test) ($F[1,118] = 8.05$, $p = 0.0053$), although there was no significant main effect of group ($F[1,118] = 2.39$, $p = 0.12$) and task condition ($F[1,118] = 0.23$, $p = 0.63$). *Post hoc* analysis revealed that CV during test session in the H_2O group was significantly smaller than the CV during the training session in the same group ($p = 0.020$, simple main effect test) and the CV during test in the GLU group ($p = 0.0024$, simple main effect test). However, there was no significant difference in CV between training and test sessions in the GLU group ($p = 0.098$, simple main effect test) nor significant difference in CVs during training session between GLU and H_2O groups ($p = 0.36$, simple main effect test). **Figure 6C** shows a statistical comparison of mean firing rates by two-way-ANOVA. The results indicated that there was no significant main effect of group ($F[1,118] = 0.08$, $p = 0.78$) and task condition ($F[1,118] = 1.57$, $p = 0.21$), or significant interaction between the group and task condition ($F[1,118] = 0.95$, $p = 0.33$). We also tested whether CV and/or mean firing rates changed across the four experimental days in the test session by two-way-ANOVAs with two factors: group (GLU vs. H_2O) and day (days 1–4). The statistical results indicated that there were no significant differences in CV and mean firing rates among the 4 days (i.e., no significant main effect of day, nor no significant interaction between day and group: data not shown). These results indicate that the firing variability of pMSNs in the dark period was maintained in the test session without oral water intake in the GLU group, while the firing variability of pMSNs was reduced in the test session in the H_2O group.

The electrode locations in each recording session were calculated based on the implanted coordinate and the total microdrive advancement (**Figure 7**), indicating that all NA neurons were recorded from the core of NA.

DISCUSSION

In the present study, we test the hypothesis that a dry-licking behavior associated with IG glucose affects NA neuronal firing using a new operant dry-licking paradigm where IG self-administration of glucose was able to induce a licking behavior, in spite of the absence of oral-intake. The present results indicate that IG glucose self-administration induced different firing pattern of NA neurons than water IG glucose self-administration.

Operant Dry Licking Behavior

In this study, a new paradigm of dry-licking behavior was used to assess the ability of IG self-administration of 0.4 M glucose to enhance licking behavior in spite of the absence of oral-intake. Daily dry-licks across 4 days in the test session were increased in the GLU group with IG self-administration of glucose, compared to the H_2O group with IG self-administration of water. Consistent with the present results, previous studies report similar results that IG nutrient without oral feedback exerts rewarding effects (Tellez et al., 2013). The present results extend previous findings; rewarding effects of IG infusion of glucose support not only licking a dry-spout but also instrumental conditioning (i.e., lever pressing upon light on).

Rewarding effects of IG infusion of glucose might be mediated by DA release in NA. Previous studies reported that IG infusion of glucose or sucrose induced DA release in NA (Ren et al., 2010). It is believed that food and water, or cues associated with them, activate DA neurons, and facilitate behaviors directed toward the acquisition of reward (Palmiter, 2007). Thus, DA might affect NA neuronal firings, which might modulate licking behaviors (Frazier and Mrejeru, 2010).

Neuronal Firing Patterns in NA

We found that CV of pMSN activity decreased in the dark period of the test session without oral intake of water, compared to that in the training session of the H_2O group, while the CV was maintained in the same test session in the GLU group.

Licking-behavior also showed similar changes; licking-counts in the light-period were decreased in the test session of the H_2O group compared to the GLU group, suggesting that the firing variability (i.e., CV) in the dark-period reflects motivational state and/or post-ingestive effect (see below in details).

The reduction of the licking behavior in the light period of the test session of the H_2O group may be associated with a reduction in DA release in NA during the dark period. Previous studies suggest a relationship between DA release and appetitive behaviors; tonic (slow) increases in DA level are involved in motivation (Niv et al., 2007; Beierholm et al., 2013; Hamid et al., 2016), seeking for reward is positively correlated with the DA level (Hamid et al., 2016), and DA modulates vigor (Beierholm et al., 2013; Hamid et al., 2016). A previous study also reported increases in DA level during delay before starting the task (Hamid et al., 2016). The present behavioral results indicated that animals performed the task less vigorously in the test session without oral intake in the H_2O group. Taken together, these results suggest that DA release might be reduced in the dark period of the test session in the H_2O group.

In the present study, firing variability (CV) of pMSNs in the dark period of the test session was decreased in the H_2O group. Previous studies suggest that DA may increase CV of activity of medial spiny neurons (MSNs) in the NA. Firing variability is associated with transition between the up-state and the down-state of MSNs (Stern et al., 1997) and the transition may be potentiated by DA (Tritsch and Sabatini, 2012). Finally, CV is defined as a good parameter in the quantal analysis of excitatory post-synaptic potentials (EPSPs) in the striatal DA-ergic transmission (Murer et al., 2002). These findings suggest that DA release increases firing variability by increasing up- and down-state transition. Furthermore, such an elevated DA level itself is involved in spontaneous fluctuation of DA release (Stuber et al., 2005), and MSN activity in NA is modulated by DA transmission (Tritsch and Sabatini, 2012), suggesting that such a DA fluctuation might also account for firing variability. Furthermore, physical behaviors were similar between GLU and H_2O groups in the dark-period of the test session, suggesting that the difference in firing variability of pMSNs was not ascribed to differences in physical behaviors between GLU and H_2O groups.

In the present study, there was no significant difference in CV across the 4 days in the test session although there was a significant main effect of group (GLU vs. H_2O groups). This suggests that the rats in the GLU group might continue to be motivated to acquire IG glucose infusion as well as water from the spout across the 4 days in the test session although lick counts were decreased in day 4. Further studies with longer experimental days might clarify changes in CV across the experimental days in the test session.

Taken together, these results suggest that decreases in firing variability of pMSNs in the H_2O group may reflect decreases in DA release in H_2O group, which consequently may decrease lick counts in test session in H_2O group. In conclusion, the present results have demonstrated that IG glucose self-administration is able to induce neuronal activation in the brain rewarding system, suggesting the role of a "gut-brain DA axis" in appetitive behaviors. However, further studies such as simultaneous recording of DA release and MSN firing is required to confirm this hypothesis.

AUTHOR CONTRIBUTIONS

EP conceived the study. EP, JM, and HisN designed the experiment. EP performed the experiment. EP and JM analyzed data and wrote the paper. HisN, HirN, YT, and TO revised the paper. All the authors discussed the results and commented on the manuscript, and read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 23 August 2017
doi: 10.3389/fphar.2017.00581

Experience-Related Changes in Place Cell Responses to New Sensory Configuration That Does Not Occur in the Natural Environment in the Rat Hippocampus

*Dan Zou[1,2], Hiroshi Nishimaru[1], Jumpei Matsumoto[1], Yusaku Takamura[1], Taketoshi Ono[1] and Hisao Nishijo[1]**

[1] System Emotional Science, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan, [2] Department of Pathophysiology, Shenyang Medical College, Shenyang, China

The hippocampal formation (HF) is implicated in a comparator that detects sensory conflict (mismatch) among convergent inputs. This suggests that new place cells encoding the new configuration with sensory mismatch develop after the HF learns to accept the new configuration as a match. To investigate this issue, HF CA1 place cell activity in rats was analyzed after the adaptation of the rats to the same sensory mismatch condition. The rats were placed on a treadmill on a stage that was translocated in a figure 8-shaped pathway. We recorded HF neuronal activities under three conditions; (1) an initial control session, in which both the stage and the treadmill moved forward, (2) a backward (mismatch) session, in which the stage was translocated backward while the rats locomoted forward on the treadmill, and (3) the second control session. Of the 161 HF neurons, 56 place-differential activities were recorded from the HF CA1 subfield. These place-differential activities were categorized into four types; forward-related, backward-related, both-translocation-related, and session-dependent. Forward-related activities showed predominant spatial firings in the forward sessions, while backward-related activities showed predominant spatial firings in the backward sessions. Both-translocation-related activities showed consistent spatial firings in both the forward and backward conditions. On the other hand, session-dependent activities showed different spatial firings across the sessions. Detailed analyses of the place fields indicated that mean place field sizes were larger in the forward-related, backward-related, and both-translocation-related activities than in the session-dependent activities. Furthermore, firing rate distributions in the place fields were negatively skewed and asymmetric, which is similar to place field changes that occur after repeated experience. These results demonstrate that the HF encodes a naturally impossible new configuration of sensory inputs after adaptation, suggesting that the HF is capable of updating its stored memory to accept a new configuration as a match by repeated experience.

Keywords: hippocampus, place cell, sensory conflict, backward translocation, mismatch cell

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Edited by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico

Reviewed by:
Yoshio Sakurai, Doshisha University, Japan
Fabio Cardoso Cruz, Federal University of São Paulo, Brazil

***Correspondence:**
Hisao Nishijo
nishijo@med.u-toyama.ac.jp

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *22 June 2017*
Accepted: *11 August 2017*
Published: *23 August 2017*

Citation:
Zou D, Nishimaru H, Matsumoto J, Takamura Y, Ono T and Nishijo H (2017) Experience-Related Changes in Place Cell Responses to New Sensory Configuration That Does Not Occur in the Natural Environment in the Rat Hippocampus. Front. Pharmacol. 8:581. doi: 10.3389/fphar.2017.00581

INTRODUCTION

The hippocampal formation (HF) is involved in encoding and retrieval of episodic memory (Squire et al., 1993; Schacter et al., 1996; Vargha-Khadem et al., 1997; Frank et al., 2000; Wood et al., 2000; Yancey and Phelps, 2001; Ferbinteanu and Shapiro, 2003; Eichenbaum, 2004). Previous lesion and neurophysiological studies using rodents and humans suggest that the HF does not encode new sensory stimuli themselves, but does encode new temporal or spatial combinations of each sensory stimulus (Honey et al., 1998; Wan et al., 1999; Eichenbaum, 2000; Takakura et al., 2003; Furusawa et al., 2006). Consistent with these findings, recent studies suggest that, to encode new information, the HF may function as a comparator to detect differences (i.e., mismatch) between internal representation in the HF and actual sensory inputs from the environment (Gray, 1982; Hasselmo and Schnell, 1994; Hasselmo and Wyble, 1997; Moser and Paulsen, 2001; Vinogradova, 2001; Havekes and Timmer, 2007; Kumaran and Maguire, 2007; Takahashi and Sakurai, 2009; Zou et al., 2009). Thus, the HF holds internal representation of the environmental information that will be compared with incoming information (Gray, 1982; Vinogradova, 2001; Kumaran and Maguire, 2007). Consistently, the environmental information is stored as a map-like representation in the HF (O'Keefe and Nadel, 1978). Place cells, the activity of which increases in a specific location in the environment, may represent this information (O'Keefe and Dostrovsky, 1971; McNaughton et al., 1983; Eichenbaum et al., 1987; Wiener et al., 1989; Kobayashi et al., 1997). Furthermore, previous neurophysiological studies reported that the HF place-related neurons represented configuration of various information encountered during navigation (Ono et al., 1993; Dayawansa et al., 2006; Ho et al., 2008; Hori et al., 2011). Another type of HF neurons has been reported (mismatch cells). Mismatch cells are active when the rats find a novel stimulus or fail to find a familiar stimulus at a particular location (O'Keefe and Nadel, 1978; Otto and Eichenbaum, 1992). These two types of the HF neurons might be important components in the HF neural circuits for a comparator that detects sensory mismatch.

Sensory mismatch has been implicated in motion sickness (Kohl, 1983; de Graaf et al., 1998). Vestibular organs including semicircular canals and otolith organs play an important role to induce motion sickness (Igarashi, 1990). When convergent sensory-motor inputs including vestibular, visual, and somatosensory inputs as well as motor efferent copies do not match the expected sensory patterns in the HF store, spatial orientation is disturbed, inducing motion sickness. Unit recording studies in zero-gravity parabolic flight and in the Space Shuttle reported that activity of HF place cells as well as head direction cells in the thalamus, which sends directional information to the HF, was abnormal in such environment, where humans often suffer from motion sickness (Knierim et al., 2000, 2003; Taube et al., 2004). Furthermore, previous behavioral studies with pharmacological manipulation in the HF suggest that the HF is involved in motion sickness in rodents (Horii et al., 1994; Uno et al., 2000). These findings suggest that this neural mismatch signal may be generated in the HF. Furthermore, training in mismatch condition ameliorates motion sickness (Stern et al., 1989; Stroud et al., 2005). This further suggests that the neural store in the HF is also updated by the neural mismatch signals to register a new configuration of sensory-motor inputs. When the neural store in the HF is updated, the HF comparator accepts the mismatch condition as the match, and the learning (i.e., adaptation/habituation) processes are terminated (Takeda et al., 2001).

Previous studies suggest that theta rhythm is implicated in both the encoding and retrieval of information in the HF (Hasselmo et al., 2002; Manns et al., 2007b; Terada et al., 2017) and also in modulation of HF synaptic current during learning (Brankack et al., 1993; Wyble et al., 2000; Orr et al., 2001), suggesting that HF theta waves might reflect the activity of a HF comparator during navigation (Zou et al., 2009). In our previous studies, to analyze HF theta rhythm and thalamic head direction cell activity in a mismatch condition, rats locomoted on a treadmill that was translocated along a figure 8-shaped track by a motion stage (Zou et al., 2009; Enkhjargal et al., 2014). In a mismatch condition, although rats locomoted forward on the treadmill, the treadmill itself was translocated backward (Zou et al., 2009; Enkhjargal et al., 2014). In this mismatch condition, idiothetic sensory inputs (optic flow, vestibular inputs, and proprioceptive inputs or motor efferent copies) contradicted each other (i.e., mismatched); movement direction indicated by the proprioceptive inputs and/or motor efferent copies during locomotion did not match that indicated by the visual-vestibular inputs. Both types of information (i.e., locomotion-related and vestibular inputs) are reported to be indispensable for HF activity (Hirase et al., 1999; Dayawansa et al., 2006; Russell et al., 2006; Lu and Bilkey, 2009). The results in the mismatch condition indicated that sensory conflict (mismatch) among idiothetic sensory inputs elevated HF theta activity, while theta activity gradually decreased after repeated exposure to the conflict (Zou et al., 2009). This suggests that new place cells encoding a new configuration of convergent inputs to the HF are formed in the HF after repeated experience.

Among the HF neural circuits, those in the CA1 area play a critical role in stimulus encoding as well as retrieval (Manns et al., 2007b), suggesting that a new configuration of convergent inputs could be represented in this area. Consistent with the idea, a recent neurophysiological study reported that some HF CA1 neurons showed specific spatial responses only during backward ambulation, but not during forward ambulation, while some other HF CA1 neurons showed spatial responses only during forward ambulation (Maurer et al., 2014). However, another recent study reported that HF CA1 neurons showed similar spatial responses during forward and backward translocation on a treadmill (Cei et al., 2014). The difference between these two studies might be ascribed to the difference in movement patterns of extremities between the two studies (reverse ambulation vs. forward locomotion on a treadmill during backward translocation) (Cei et al., 2014). However, there might be another possibility; the rats were well-trained with backward ambulation in the former study (Maurer et al., 2014), while the rats were trained only in the forward condition in the latter study (Cei et al., 2014). Therefore, we hypothesized that the differences in spatial responses between the two studies

might be attributed to the differences in experiences (training) in the backward condition, and that HF neurons would develop different spatial responses between the forward and backward translocation on a treadmill after being well-trained.

In the present study, to investigate whether the HF CA1 place cells were capable of encoding mismatched information that does not occur naturally after being well-trained, we recorded the HF CA1 neuronal activity from rats that were well-trained in a set-up that replicated the mismatch conditions of the previous study (Zou et al., 2009).

MATERIALS AND METHODS

Subjects

Seven Wistar male rats weighing 200–300 g were used. The animals were similarly treated according to our previous protocols (Dayawansa et al., 2006; Zou et al., 2009; Enkhjargal et al., 2014). They were individually housed in cages controlled at a constant temperature (20 ± 2°C) with free access to water and laboratory chow. All rats were treated in strict compliance with the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Guidelines for the Care and Use of Laboratory Animals of the University of Toyama. The study was approved by the Committee for Animal Experiments and Ethics at the University of Toyama (Permit number: S-2009MED-25).

Surgery

The same surgical procedures were used as those in our previous studies (Dayawansa et al., 2006; Zou et al., 2009; Enkhjargal et al., 2014). Briefly, the rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.). First, several stainless screws were implanted in the bone as anchors. One of the screws over the cortex near the HF was used as a ground electrode. Then, a cranioplastic cap was molded on the skull according to our previous studies (Nishijo and Norgren, 1990; Uwano et al., 1995). After the surgery, an antibiotic (gentamicine sulfate) was administered topically and systemically (2 mg, i.m.). This cranioplastic cap was used as artificial earbars; the cranioplastic cap can be painlessly fixed in the stereotaxic apparatus on the treadmill. After 1 week of recovery, the rats were trained in a navigation task on a treadmill (see "Training and behavioral testing" in detail).

After training, the rats were reanesthetized, and a hole (3–5 mm diameter) for semi-chronic recording was drilled through the cranioplastic and the underlying skull over the cortex near the HF (A, −2.0 to −4.0 from bregma: L, 2.0 to 6.0). The exposed dura was removed, and the hole was covered with a sterile Teflon sheet and sealed with epoxy glue for later neuronal recording.

Experimental Setup and Tasks

The same apparatus and tasks were used as those in our previous studies (Dayawansa et al., 2006; Zou et al., 2009; Enkhjargal et al., 2014). Briefly, a transparent plastic enclosure for semi-chronic recording (Nishijo and Norgren, 1990) was placed on a treadmill, which was fixed on a stereotaxic apparatus. The enclosure lacked a floor so that the rats could locomote on the treadmill. The stereotaxic apparatus was further attached to the motion stage (**Figure 1A**). The cranioplastic cap on the rat's head was painlessly fixed to a stereotaxic frame on the motion stage. The motion stage was translocated horizontally by belts with two motors (THK Co., Kanazawa, Japan). Another motor, attached to the base of the motion stage, rotated the motion stage so that the rat faced in the direction of translocation.

In a forward condition (**Figure 1C**), the motion stage was translocated between Places I and II in a figure 8-shaped pathway consisting of Routes 1 and 2 at the speed of 20 cm/s. During this translocation, the rats always faced toward the direction of the tangent of the translocation routes to imitate directional changes in natural navigation. The treadmill was also operated at the same speed (20 cm/s) as the translocation speed of the stage, which reliably induced locomotion of the rat. In Route 1 (**Figure 1Ca**), the rat was translocated from Place I to Place II, and in Route 2 (**Figure 1Cb**), from Place II to Place I. Thus, Routes 1 and 2 included a common central stem in the figure 8-shaped pathway. At the Places I and II, the motion stage paused, and a delayed stimulus-response association (DSR) task was imposed (see below in detail). After the DSR task, the motion stage was rotated so that the rat faced in the direction of translocation in Routes 1 and 2 before translocation.

In a different condition (backward condition), the motion stage was initially rotated by 180°, and then translocated in the same way (**Figure 1D**). In this backward condition, although the rat locomoted forward on the treadmill, the motion stage was translocated backward (opposite to the rat's locomoting direction) (i.e., mismatch condition).

In both the forward and backward conditions, the stage stopped at the end of the pathways (i.e., Places I and II), where the rats were required to perform the DSR task (**Figures 1Ba,b**). In the DSR task, the rats could acquire rewards, which was important to keep the rats locomoting on the treadmill throughout the recording sessions. At Place I, the DSR task started by the 530-Hz tone for 0.5 s (**Figure 1Ba**). After a 1.5 s delay, the treadmill was operated at 20 cm/s for 3.0 s twice with an intervening 2.0 s interval in which the treadmill was stopped. The rats reliably locomoted without reward during these 3.0 s runs. At Place II, the task similarly started by the same 530-Hz tone, and after a 1.5 s delay the treadmill was operated at 20 cm/s for 3.0 s (**Figure 1Bb**). After the second delay of 2.0 s, the tube was protruded close to the rat's mouth for 2.0 s. The rat could ingest a water reward, if it licked the tube during this period. Water licking was detected by a touch sensor connected to the tube.

Training and Behavioral Testing

The same training procedures were used as those in our previous studies (Zou et al., 2009; Enkhjargal et al., 2014). Briefly, the rats were acclimated to being placed for short periods in the plastic restraining enclosure on the motion stage before and after the surgery. Then, the rats were trained to perform the DSR task under a 24-h water-deprivation regimen. Second, after the rats could constantly perform the DRS task, they were trained to locomote on the treadmill while the motion stage

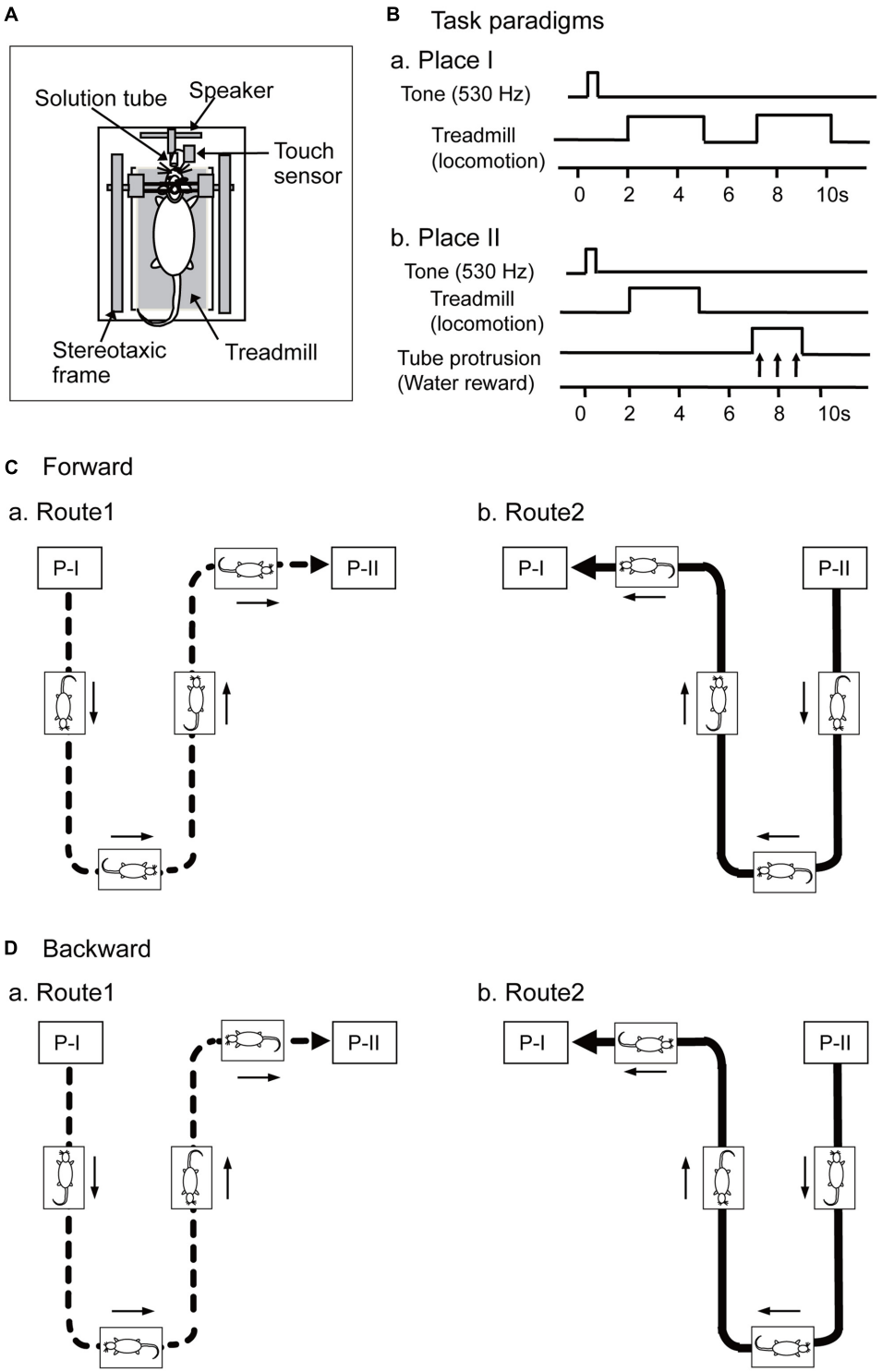

FIGURE 1 | Schema of the experimental paradigms. **(A)** Set up of a stereotaxic apparatus and a treadmill that were attached to a motion stage, and the rat was placed on the treadmill inside a spacious transparent plastic enclosure. **(B)** Paradigms of a delayed stimulus-response association (DSR) task that was carried out at Places I and II. At Place I **(Ba)**, the task was initiated by a cue tone and followed by two periods of 3.0 s during which the treadmill rotated. At Place II **(Bb)**, the task was initiated by the same cue tone and followed by a 3.0 s period of treadmill rotation and a 2.0 s period of tube protrusion. The tone and the following treadmill operation were separated by 1.5 s intervals, while the two reinforcements were separated by 2.0 s intervals. **(C,D)** Movements of the motion stage in forward (**C**, forward sessions) and backward translocation (**D**, backward sessions). The motion stage moved on a Figure 8-shaped route consisting of Routes 1 (a) and 2 (b), and the start point of each route was designated as Places I and II, respectively. Arrows indicate movement direction of the motion stage.

was translocated in the forward condition for 2 weeks. The rats usually ingested 20–30 ml of water in the restrainer. If the rat failed to drink a total volume of 30 ml water, it was allowed to drink the remainder in its home cage. Our previous studies indicated that the rats well learned the tasks under the similar water-deprivation regimens (Dayawansa et al., 2006; Zou et al., 2009; Enkhjargal et al., 2014).

Finally, the rats were trained in the forward and backward conditions for 5 days. In this final training, a total of three sessions/day were conducted. Each session consisted of three laps of translocation. After each lap (Routes 1 and 2) of translocation, each three trials of the DSR task were imposed at Places I and II, respectively. In the 1st and 3rd sessions, the rats performed the task in the forward condition, while they did the task in the backward condition in the 2nd session. After these trainings, activity of the neurons was recorded from the HF CA1 area under the same protocol with three sessions; (1) an initial control session in the forward condition, in which both the stage and the treadmill moved forward, (2) a backward (mismatch) session, in which the stage was translocated backward while the rats locomoted forward on the treadmill, and (3) the second forward condition.

Neuronal Recordings and Unit Isolation

The same neurophysiological procedures were used as those in our previous study (Dayawansa et al., 2006). Briefly, after the rat was placed in the stereotaxic apparatus on the motion stage, the Teflon sheet was removed, and a glass-insulated tungsten microelectrode ($Z = 1.0$–1.5 MΩ at 1 KHz; tip diameter < 5 μm) was stereotaxically inserted into various parts of the HF CA1 area. The neuronal signals, triggers for the tone, tube protrusion, and licking, and the X–Y coordinates of the motion stage were digitized and stored in a computer. The Offline Sorter program (Plexon, Dallas, TX, United States) sorted neuronal activities into single units by their waveform components. Superimposed wave forms of the sorted units were inspected to check the invariability of the sorted units throughout the recording sessions (see below in detail). Then, the sorted unit activities were transferred to the NeuroExplorer program (Nex Technology, Littleton, MA, United States) for further analysis.

Examples of superimposed spike waves of a HF neuron and the autocorrelograms of the neuronal spikes are shown in Supplementary Figure 1. We carefully inspected data throughout the sessions and those before and after the sessions. The data indicated that the superimposed waveforms (a) and various waveform parameters in cluster cutting projections (data not shown) were similar across the sessions. The autocorrelograms (b) showed that a refractory period of the CA1 neuron was 2–3 ms throughout the recording sessions, suggesting that these spikes were recorded from a single neuron.

When HF neuronal activities were isolated, their activities were recorded while the rats performed the task in the three sessions (see "behavioral testing" in detail). In these experimental conditions, effects of sensory mismatch were analyzed. Every condition always started at Place I in Route 1.

Analysis of Place-Differential Activity

Place-differential activity was analyzed according to our previous study that used the same experimental setup and tasks (Dayawansa et al., 2006). Briefly, each route was divided into 56 successive pixels, and the firing rate maps in Routes 1 and 2 were separately constructed in each session. Place-differential activities were separately defined in Routes 1 and 2. First, the firing rate maps in each route were created by a smoothing method, in which the smoothed firing rate of a given pixel was defined as the mean of three pixels (the given pixel and the two adjoining pixels) (Dayawansa et al., 2006). Second, all pixels with an increase in the mean firing rate, which was defined as a firing rate greater than 2.0 times the grand mean firing rate of a given neuron in either Routes 1 or 2, were identified (Muller and Kubie, 1987; Kobayashi et al., 1997; Dayawansa et al., 2006). A place-differential activity was classified as such if it had at least three adjacent pixels with an increase in the mean firing rate (i.e., place field). This place cell analysis was separately carried out in individual sessions; 1st forward, 2nd backward, and 3rd control sessions. Only the place-differential activities with place field(s) in at least one of the three sessions were further analyzed.

To assess changes in spatial firing patterns across the sessions, we computed pixel-to-pixel correlation coefficients (r) of firing-rate distributions between the first (control) and the following sessions (Dayawansa et al., 2006).

Classification of Place-Differential Activities

The place-differential activities were initially classified based on the place fields and peak firing rates within the place fields. When the HF neuronal activities displayed place fields in the backward sessions and if the peak firing rates in the place fields of the backward session were more than four times of those in the control sessions in corresponding route, the activities were defined as backward-related regardless of correlation coefficients. When the HF neuronal activities displayed place fields at least in the 1st and/or last control sessions and if the peak firing rates in the place field(s) of the forward sessions were four times larger than those in the backward sessions in corresponding route, the activities were defined as forward-related regardless of the correlation coefficients in this route. The remaining place-differential activities that displayed place fields in both the forward and backward sessions were defined as both-translocation-related activities.

These forward-related and both-translocation-related activities were further grouped into two subcategories based on correlation coefficients. For forward-related activities, if correlation coefficients between 1st and 3rd forward sessions in a given route (Route 1 or Route 2) were larger than 0.4, the place-differential activities were defined as session-independent for that route. For both-translocation-related activities, if correlation coefficients between 1st and 3rd forward sessions and those between the 1st forward and 2nd backward sessions in a given route (Route 1 or Route 2) were larger than 0.4, the place-differential activities were defined as session-independent

for that route. If one of the correlation coefficients did not reach 0.4, those activities were defined as session-dependent activities.

Analyses of the Place Fields

Previous studies reported experience-dependent asymmetric expansion of place fields (Mehta et al., 1997, 2000; Lee et al., 2004). We similarly analyzed the place field expansion. First, place field sizes were simply analyzed by counting number of pixels within the place fields. Second, to analyze the firing rate distribution of the place field, skewness of firing rate distribution within the place field was computed (Mehta et al., 2000). Third, since skewness does not necessarily refer to asymmetry of spatial distribution of the firing rates in terms of movement direction of the rats, asymmetry of firing rate distribution within the place field was analyzed in terms of movement direction of the rats. In this analysis, the location of the center of the mass of the firing rate distribution within the place field was computed. Then, the place field was divided into two parts by the center of the mass. The place field asymmetry index was defined as difference in the number of the pixels between the two parts (i.e., the number of the pixels in the first part of the place field along the movement direction minus the number of the pixels in the second part of the place field).

Place field sizes and place field asymmetry index were compared by one way analysis of variance (ANOVA) and following *post hoc* comparisons (Fisher LSD test; $p < 0.05$) among the four types of the place-differential activities (forward-related, backward-related, both-translocation-related, and session-dependent activities).

Histology

The same histological procedures were used as those in our previous study (Dayawansa et al., 2006). Briefly, upon completion of all the experiments, each rat was anesthetized with pentobarbital and several small electrolytic lesions were stereotaxically made around the recorded sites. The rats were then perfused, and the brains were removed and cut into serial 50 μm frontal sections. The brain sections were stained with Cresyl Violet. All marking and stimulation sites were then carefully verified microscopically. Positions of place-differential activities were sterotaxically located on the real tissue sections in each animal. Finally the recording sites were re-plotted on the corresponding sections on the atlas of Paxinos and Watson (1986).

RESULTS

The 161 HF CA1 neurons (complex spike cells) were recorded under three conditions; (1) initial control sessions, in which forward translocation with locomotion and the tasks were imposed, (2) backward sessions, in which backward translocation with locomotion and the tasks were imposed and (3) the last control sessions. Each neuron was recorded for three complete laps of translocation during each session. Of the 161 neurons, 56 place-differential activities were recorded from the HF CA1 subfield.

The place differential activities were categorized into four types; forward-related, backward-related, both-translocation-related, and session-dependent (**Table 1**). Of these, all forward-related and both-translocation-related activities displayed place fields with high similarity (i.e., $r > 0.4$) between the first and last control sessions in Route1, Route 2, or both. All of the 4 types of the HF place-differential activities were recorded from each rat, and percentages of each activity type were not different among the rats (data not shown). Furthermore, HF neurons with these four types of activities did not show activity change during the DSR task (data not shown).

Forward-Related Activities

In Route 1, 12 activities (21.4%) showed place fields with high correlation coefficients between the 1st and 3rd forward sessions, but did not show place fields in the 2nd backward session. However, 2 of them showed high correlation coefficients between the 1st control and 2nd backward sessions. In Route 2, 17 activities (30.4%) showed place fields with high correlation coefficients between the 1st and 3rd forward sessions, but did not show place fields in the 2nd backward session. The mean correlation coefficients of these 29 activities between the first and last control session [0.64 ± 0.03 (mean ± SEM)] was significantly larger than that between the 1st and 2nd sessions (-0.001 ± 0.05) (Wilcoxon signed rank sum test, $P < 0.001$).

Figures 2A–C shows the representative data of a HF place-differential activity that showed a spatial firing pattern dependent on forward translocation. This activity showed stable place fields in both the 1st and the last control sessions (forward translocation) on Route 1 ($r = 0.76$; 1st vs. 3rd) and Route 2 ($r = 0.83$; 1st vs. 3rd). It is noted that the peak firing rates in the place fields in Routes 1 and 2 decreased in the 2nd backward session to a level less than one fourth of those in the 1st control trials, although the correlation coefficient between the 1st and the 2nd sessions in Route 1 was relatively high ($r = 0.58$). An example of a peri-event time histogram of the neuronal activity during forward translocation in Route 2 in the 1st session is shown in

TABLE 1 | Response characteristics of the 56 hippocampal formation (HF) place-differential activities in Routes 1 and/or 2.

Category	Route 1	Route 2
Forward-related	12	17
Backward-related	14	5
Both-translocati on-related	11	3
Session-dependent	18	27
No place fields	1	4
Total	56	56

The table indicates the number of the HF activities, response characteristic of which matched the definition of each category in each route. Note that place-differential activities were separately defined in each route, and some HF neurons showed spatial activities in both of the routes in different or same categories.

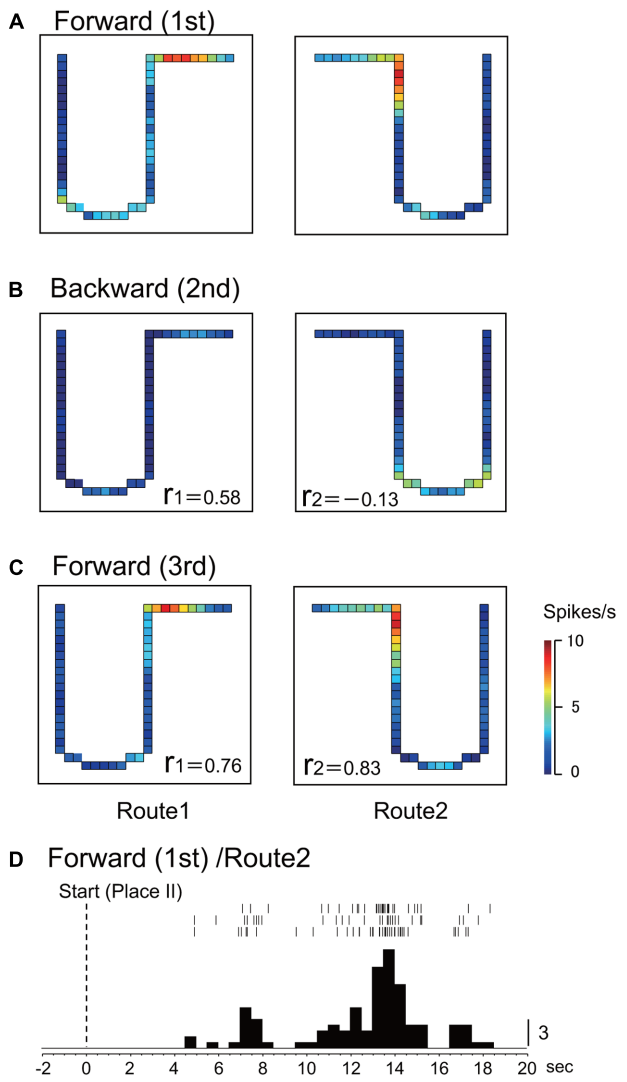

FIGURE 2 | An example of a forward-related place-differential activity. **(A–C)** This activity displayed place fields in both Routes 1 and 2 in the 1st **(A)** and the 3rd **(C)** forward sessions, but not in the 2nd backward sessions **(B)**. *r*, correlation coefficients between firing-rate distributions in the first control session and the following given session in Route 1 (r 1) and Route 2 (r 2), respectively. The color of each pixel indicates neuronal activity calibrated at the right bottom (spikes/s). **(D)** Peri-event time histogram of the neuronal activity during forward translocation in Route 2 in the 1st session. The neuronal activity increased around 13 s after the start from Place II. A histogram shows summed neuronal activity. Each histogram bin, 500 ms; calibration at right of each histogram, number of spikes in each bin. The histograms were aligned with start of translocation.

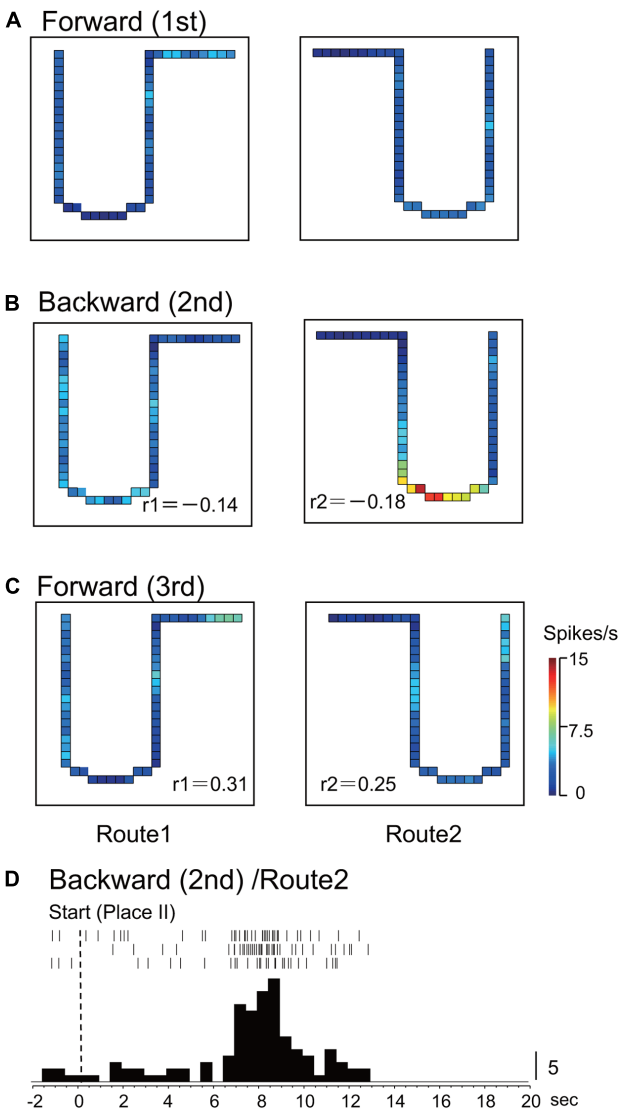

FIGURE 3 | An example of a backward-related place-differential activity. **(A–C)** This activity displayed a place field in Route 2 only in the backward session **(B)**, but not in the forward sessions **(A,C)**. **(D)** Peri-event time histogram of the neuronal activity during backward translocation in Route 2 in the 2nd session. The neuronal activity increased around 8 s after the start from Place II. Other conventions are the same as in **Figure 2**.

Figure 2D. The neuronal activity increased around 13 s after the start from Place II.

Backward-Related Activities

In Route 1, 14 activities (25.0%) did not display significant place fields in the 1st and the 3rd forward sessions, but place fields appeared in the backward session. In Route 2, 5 activities (8.9%) did so in the same way. **Figures 3**, **4** illustrate the examples of HF place-differential activities that displayed place fields in Route 2 (**Figure 3**) and the common stem of Routes 1 and 2 (**Figure 4**) only in the backward session, respectively. Since these activities displayed place fields only in the 2nd session, the correlation coefficients between the 1st and the 2nd sessions were low [−0.18 (Route 2) in **Figure 3**; 0.28 (Route 1) and −0.19 (Route 2) in **Figure 4**]. Examples of peri-event time histograms of the neuronal activity during backward translocation in Route 2 in the 2nd session is shown in **Figures 3D**, **4D**. The neuronal activity increased around 8 and 13 s after the start from Place II in **Figures 3D**, **4D**, respectively.

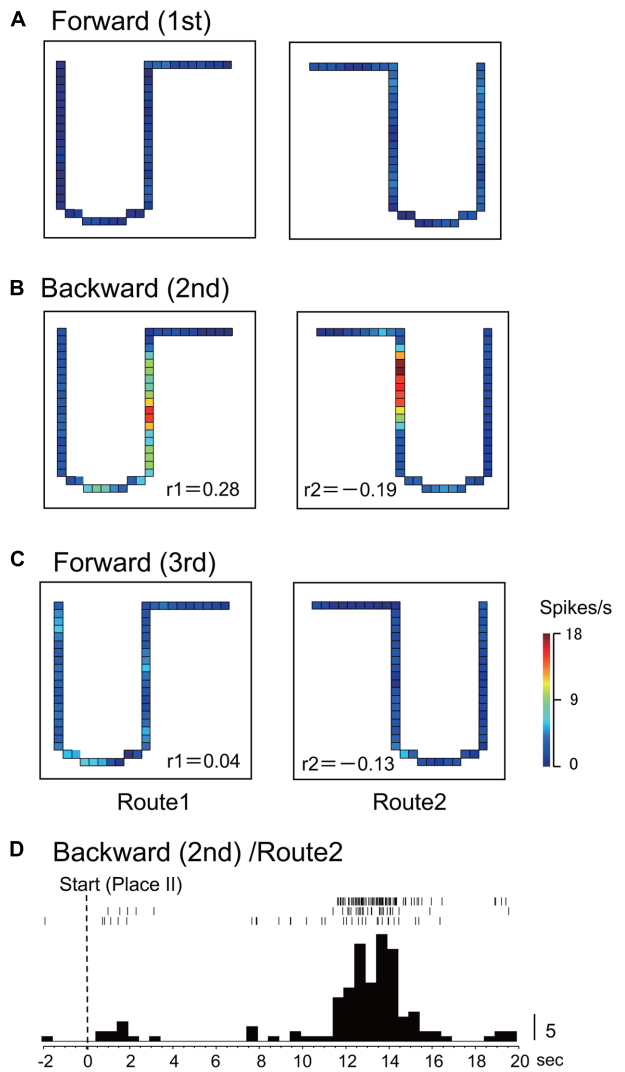

FIGURE 4 | Another example of a backward-related place-differential activity. **(A–C)** This activity displayed place fields in both Routes 1 and 2 only in the backward session **(B)**, but not in the forward sessions **(A,C)**. **(D)** Peri-event time histogram of the neuronal activity during backward translocation in Route 2 in the 2nd session. The neuronal activity increased around 13 s after the start from Place II. Other conventions are the same as in **Figure 2**.

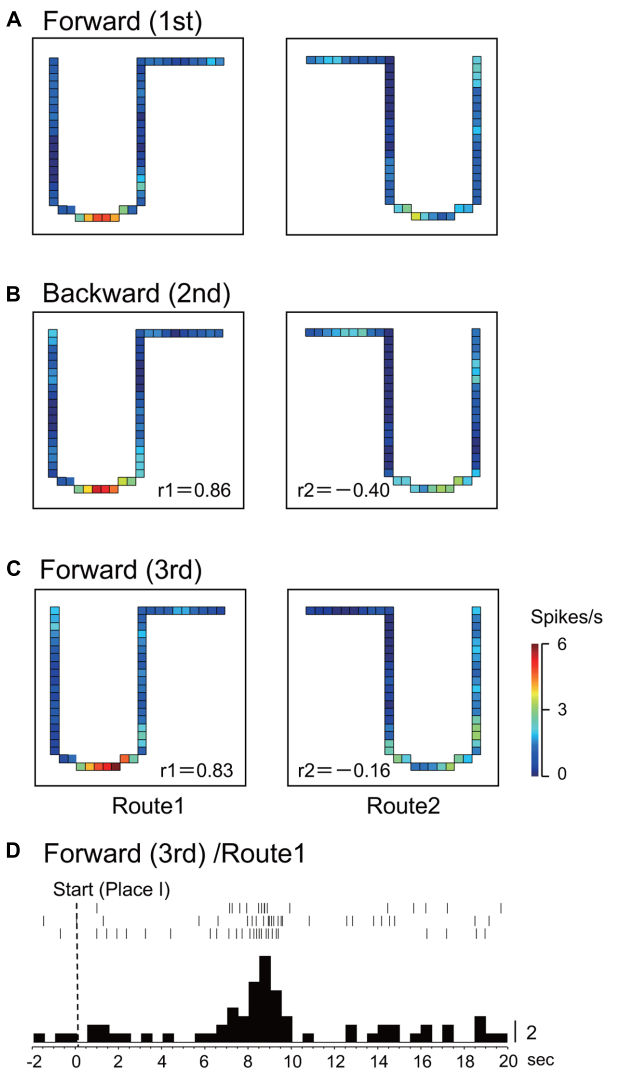

FIGURE 5 | An example of a both-translocation-related place-differential activity. **(A–C)** The place fields were consistently observed across the three sessions in Route 1. **(D)** Peri-event time histogram of the neuronal activity during forward translocation in Route 1 in the 3rd session. The neuronal activity increased around 9 s after the start from Place I. Other conventions are the same as in **Figure 2**.

Both-Translocation-Related Activities

In Route 1, 11 activities (19.6%) displayed place fields in each session. Furthermore, Pearson's correlation coefficients between the 1st and the 3rd forward sessions as well as those between the 1st forward and the 2nd backward sessions were larger than 0.4 in these activities. In Route 2, 3 activities (5.3%) displayed the place fields in the same way. The mean correlation coefficients (0.61 ± 0.03) between the 1st and the 3rd forward sessions were comparable to those (0.6 ± 0.04) between the 1st and the 2nd session (Wilcoxon signed rank sum test, $P > 0.05$).

Figure 5 illustrates an example of a place-differential activity showing stable place fields in the three sessions in Route 1. This activity displayed place fields in both the 1st and the 2nd sessions. Furthermore, the place fields did not change across the sessions ($r = 0.86$, 1st forward vs. 2nd backward sessions; $r = 0.83$, 1st and 3rd forward sessions). An example of a peri-event time histogram of the neuronal activity during forward translocation in Route 1 in the 3rd session is shown in **Figure 5D**. The neuronal activity increased around 9 s after the start from Place I.

Session-Dependent Activities

Of these 56 place-differential activities, 12 (21.4%) showed session-dependent spatial firing patterns, which remapped the place field, in both routes across sessions. The other 6 (10.7%) activities showed a session-dependent spatial firing pattern across

sessions in Route 1, while other 15 (26.8%) showed session-dependent spatial firing patterns across sessions in Route 2. **Figure 6** illustrates an example of a place-differential activity showing session-dependent place fields in Route 1. The activity displayed different place fields across the sessions, which resulted in low correlation coefficients between the 1st forward and the 2nd backward sessions ($r = -0.15$), and between the 1st and 3rd forward sessions ($r = -0.12$). However, this activity also displayed a place field only in the 2nd backward session in Route 2 (backward-related activity). An example of a peri-event time histogram of the neuronal activity during backward translocation in Route 2 in the 2nd session is shown in **Figure 6D**. The neuronal activity increased around 6 s after the start from Place II.

Comparison of the Place Fields

Figure 7A illustrates mean sizes of the place fields per route in each type of the place-differential activities. One way analysis of variance (ANOVA) indicated that there was a significant difference among the groups [$F(3,248) = 12.98$, $P < 0.001$]. *Post hoc* tests by Fisher LSD test indicated that the mean place field sizes were larger in the forward-related, backward-related, and both-translocation-related activities than that of session-dependent activities ($P < 0.01$). In the analysis of skewness, most place-differential activities showed negative values; mean skewness in each type was -0.66 ± 0.08 (forward-related), -0.50 ± 0.07 (backward-related), -0.4995 ± 0.07 (both-translocation-related), and -0.63 ± 0.05 (session-dependent), respectively. However, there was no significant difference in skewness among the 4 types [$F(3,248) = 1.008$, $P > 0.05$].

Figure 7B illustrates mean place field asymmetry index for each type of the place-differential activities. One way analysis of variance (ANOVA) indicated that there was a significant difference among the groups [$F(3,248) = 5.160$, $P < 0.02$]. *Post hoc* tests by Fisher LSD test indicated that the mean place field asymmetry indices were larger in the forward-related, backward-related, and both-translocation-related activities than that of session-dependent activities ($P < 0.05$). Positive values of place field asymmetry indices indicate that the center of mass of firing distribution was located in the latter half of the place fields according to its definition and the firing rate gradually increased from the entrance of the place field until the center of the mass, then relatively and suddenly returned to the baseline level when the rat left the place field.

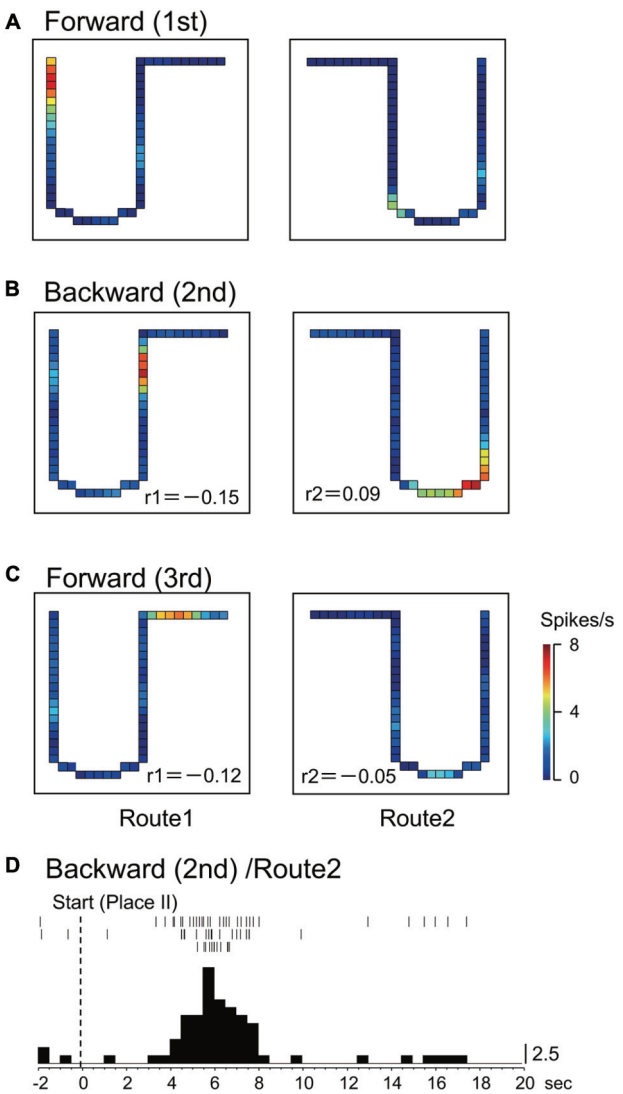

FIGURE 6 | A HF place-differential activity with session-dependent spatial firing patterns under the three sessions in Route 1 (session-dependent). **(A–C)** This activity also displayed backward-related spatial firing patterns in Route 2. **(D)** Peri-event time histogram of the neuronal activity during backward translocation in Route 2 in the 2nd session. The neuronal activity increased around 6 s after the start from Place II. Other conventions are the same as in **Figure 2**.

Locations of Place-Differential Activities

Figures 8A–E shows recording sites of the place-differential activities. These sites were stereotaxically computed from small lesions made in the HF after recording. An example of a lesion is shown in **Figure 8F**. All the HF neurons with place-differential activities were located in the CA1 area.

DISCUSSION

We investigated whether HF place cells could encode new configuration of conflicting sensory inputs after repeated exposure to the conflicting environment. Consistent with our hypothesis that the differences in spatial responses between the two studies (Cei et al., 2014; Maurer et al., 2014) might be attributed to the differences in experiences (training) in the backward condition, we found forward- and backward-related activities that were active only in the forward and backward translocation in rats with repeated experience, respectively, although locomotion was the same in these two translocations. Furthermore, the place fields of these HF activities showed experience-dependent changes in skewness. These results are discussed in terms of learning-related synaptic modification by repeated experience (see below).

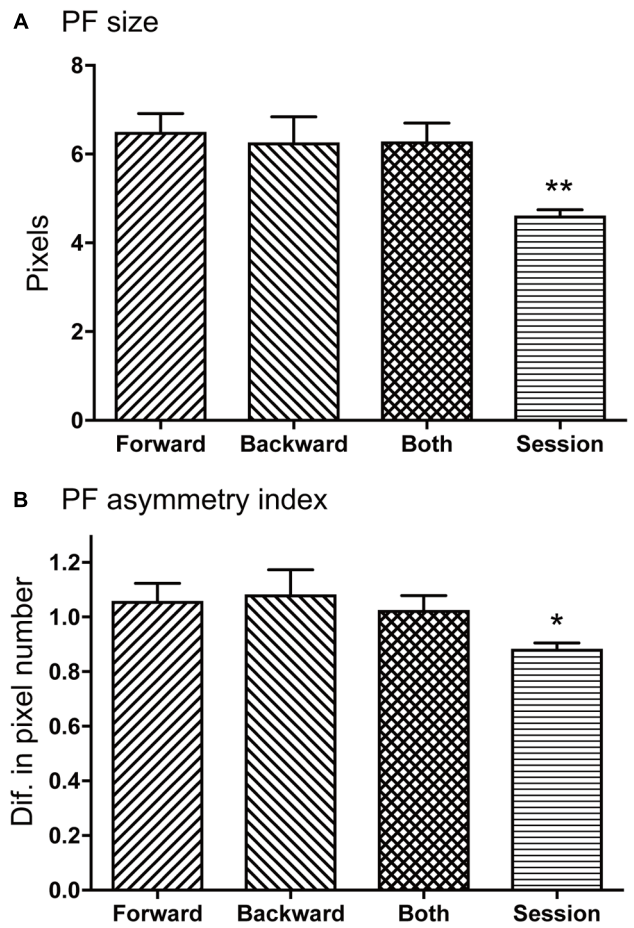

FIGURE 7 | Comparison of mean place field sizes **(A)** and place field asymmetry indices **(B)** among the four types of the HF place-differential activities. **(A,B)** The mean place field sizes were larger in the forward-related, backward-related, and both-translocation-related activities than that of session-dependent activities **(A)**, while the mean place field asymmetry indices were larger in the forward-related, backward-related, and both-translocation-related activities than that of session-dependent activities **(B)**. Ordinate in B indicates difference in number of pixels. PF, place field; Forward, forward-related activities; Backward, backward-related activities; Both, both-translocation-related activities; Session, session-dependent activities. **, * significant difference from the other three columns by $P < 0.01$ and $P < 0.05$.

Characteristics of HF Place-Differential Activities

In the present study, 4 main types of HF spatial firing patterns were observed when rats were translocated forward and backward. It should be emphasized that the rats were well-trained for more than 5 days in the backward sessions. Our previous study, which used the same set up, indicated that theta activity in the backward sessions decreased to a level comparable to that of the initial forward sessions after training for 5 days (Zou et al., 2009). These findings strongly suggest that the rats adapted well to the mismatched condition, which further suggests that these four types of neuronal activities were not mismatch cells, but rather place cells encoding a new configuration of sensory inputs. Furthermore, these place-differential activities displayed characteristics of learning-related modification in the place fields (see below in detail).

The detailed analyses of the place fields indicated that the place fields were asymmetric; skewness of the place fields was negative, and the place field asymmetry index was positive. It is noted that these asymmetric changes were consistent with the movement direction of the motion stage even in the conflicting condition (i.e., backward-related activities) in the present study. These patterns of firing distributions were similar to those reported in previous studies (Mehta et al., 1997, 2000; Lee et al., 2004), in which the size of place fields expanded by repeated experience in a direction opposite to the rat's movement. Since NMDA-dependent and temporally asymmetric long-term potentiation/long-term depression (LTP/LTD) is implicated in these types of place field changes (Mehta et al., 1997, 2000; Ekstrom et al., 2001; Lee et al., 2004), the present results suggest that HF neural circuits for the forward-related, backward-related, and both-translocation-related activities might be also modified by these NMDA-dependent and temporally asymmetrical processes. Consistent with this idea, a cross-correlation study of monkey HF neurons reported that information of spatial navigation in specific tasks is encoded by the temporally asymmetrical neural circuits connecting pyramidal neurons (Hori et al., 2011).

Our previous neurophysiological study using the same experimental set up reported that these place-differential activities were dependent on locomotion (proprioceptive and/or motor efferent copy), the task context, and vestibular sensation or visual cues such as optic flow (Dayawansa et al., 2006). These findings suggest that the forward-related and backward-related place-differential activities might play a role in encoding configuration of convergent sensory inputs (optic flow, vestibular inputs, proprioceptive and/or motor efferent copy) in the forward and backward sessions, respectively. In the present study, the activity of both-translocation-related activities increased in the same place in both the forward and backward sessions, where optic flow and vestibular sensation conflicted. These activities might encode location of the animals based on distal cues regardless of other sensations (optic flow, vestibular, and proprioceptive sensation). We also observed some HF activities that showed session-dependent spatial firings in each session, consistent with our previous study (Dayawansa et al., 2006). Consistent with the present results, another previous study indicated that the place cells sometimes spontaneously remapped in a stable environment (Ludvig, 1999). Taken together, these results suggest that the HF can encode and predict future movements that do not occur in a natural environment by repeated experience.

Effects of Learning-Related Synaptic Modification on Neural Correlates to Space

There were significant differences among the four types of place-differential activities; place field sizes were larger in

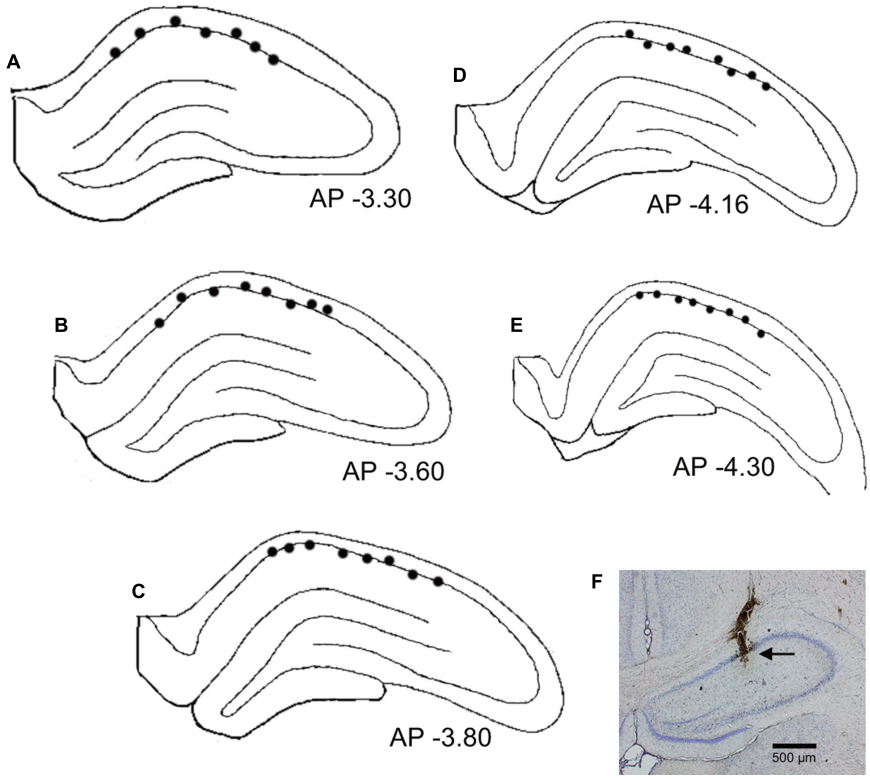

FIGURE 8 | Recording sites of the HF neurons with place-differential activities. **(A–E)** Locations of individual neurons with place-differential activities (filled circles) in each HF section. All HF neurons with place-differential activities were located in the CA1 subfields of the HF. Negative value below each section indicates distance (mm) posterior to the bregma. **(F)** An example of a coronal histological section with an electric lesion (black arrow) in the CA1 area.

the forward-related, backward-related, and both-translocation-related activities than in the session-dependent activities, and the place field asymmetry index also showed a similar trend. These findings suggest that repeated training-related changes in the place fields are more evident in the forward-related, backward-related, and both-translocation-related activities than in the session-dependent activities. These differences in training-related changes (place field asymmetry index, place field size) may account for the difference in stability of the place fields among the four types of the HF place-differential activities (see below in detail).

It is reported that stability of place cell firing fields depended on attentional demands to spatial landmarks; when the animals freely explore an environment under no task contingencies, place fields were not stable (Kentros et al., 2004; Muzzio et al., 2009). This attentional modulation was mediated through dopaminergic D1/D5 receptors (Kentros et al., 2004), and activation of D1 receptor upregulates NMDA receptor-mediated LTP (Nai et al., 2010). In the present experimental set up, the rats were translocated by the motion stage, and as a consequence, they might not strongly attend to the external landmarks. Therefore, the place fields of some HF neurons with session-dependent activities might not be stabilized by attentional modulation. Consistent with this idea, the mean place field sizes and place field asymmetry indices, which are dependent on NMDA-dependent LTP (Ekstrom et al., 2001), were smaller in the session-dependent activities. These results suggest that HF neural circuits for the forward-related, backward-related, and both-translocation-related activities might be more extensively modified by these NMDA-dependent LTP/LTD and consequently more stabilized than those for the session-dependent activities. The lack of stabilization by these NMDA-dependent processes in the session-dependent activities might account for the unstable firing distributions across the sessions. On the other hand, it is reported that HF neuronal activity gradually changes even in the same environment by encoding temporal context (Manns et al., 2007a). This mechanism might also contribute to spatial remapping in session-dependent activities.

There are some discrepancies among the studies reporting place field shifts. Some previous studies reported that the center of the mass of the place fields shifted backward after repeated trials (Mehta et al., 1997, 2000; Lee et al., 2004). However, other studies reported opposite changes; forward shift of the center of the mass of the place fields (Lee et al., 2006; Griffin et al., 2007). Nevertheless, skewness was consistently negative in all of the previous studies including the present study (i.e., the firing rates gradually increase, but abruptly decrease). These results strongly suggest that the HF neural circuits are subject to certain learning-induced synaptic modification, consistent with the recent studies (Cheng and Frank, 2008; Komorowski et al.,

2009; Tort et al., 2009; Wirth et al., 2009; Lever et al., 2010). The modes of these neural activity changes due to training-induced synaptic modification might depend on task demands for future prediction. The HF might be essential to encode the past events to form episodic memory, and based on such memory the HF is also essential to predict goals, future trajectories, and outcome of the events that have not yet occurred (Eichenbaum and Fortin, 2009; Takahashi, 2013, 2015). Some HF activities with backward shifts of the place fields in the present as well as previous studies (Mehta et al., 1997, 2000; Lee et al., 2004) might be involved in prediction of future location, while the HF neurons with forward shifts of the place fields (Lee et al., 2006; Griffin et al., 2007) might be involved more in reward prediction in which place field is expanded to future reward location. This difference in prediction demands might result in different shifts of the place fields among the studies. A recent study suggests that prediction and future planning might be executed by the interaction between the HF CA1 area and medial prefrontal cortex (Ishino et al., 2017).

Comparison with the Head-Direction Cell System

The head-direction cell system (Taube et al., 1990a,b; Taube, 1995) integrates head angular velocity to output a signal related to head direction (McNaughton et al., 1991; Blair and Sharp, 1995; Skaggs et al., 1995; Zhang, 1996). Movement direction on a radial maze strongly influences HF place cell activity (McNaughton et al., 1983). Firing properties of HF place cells are related to head-direction cell activity (Knierim et al., 1995), and the head-direction system might orient the 'cognitive map' in the HF (O'Keefe and Nadel, 1978; Knierim et al., 2000). Several studies have analyzed the activity of head direction cells when an animal was placed in a specific situation with conflicting spatial information. For example, activity of head-direction cells in the anterior dorsal thalamic nucleus was recorded under conditions where vestibular cues conflicted with optic flow (Blair and Sharp, 1996). In this mismatch condition, the majority of the cells were bound to the vestibular information. This suggests that vestibular information is essential in the neural computation for head direction (Taube, 1998). On the other hand, in well-trained rats in the same setup as in the present study, we reported four types of thalamic neurons (Enkhjargal et al., 2014); heading direction-related and movement direction-related neurons coded separately heading and movement directions regardless of direction of translocation (forward or backward), while forward and backward movement-related neurons coded movement directions only in forward and backward translocation, respectively. These results suggest that thalamic neurons can encode conflicting multiple information to identify heading and movement directions in a backward condition, if animals are well-trained.

In the present study, some HF activities (backward-related and both-translocation-related activities) were able to encode conflicting sensory/motor information in the backward session, where vestibular and visual information did not match proprioceptive information (or motor efferent copy). These results strongly suggest that HF neurons encode configuration of convergent sensory inputs, in which vestibular information is one of the sensory inputs, but not a major input. This demonstrates that the HF encodes any combination of the sensory inputs. Since HF lesions affect head direction cell activity in the thalamus (Golob and Taube, 1999), HF outputs might be integrated in the anterior thalamus (Aggleton et al., 2010). Therefore, configural information from the HF may contribute to the complex responsiveness of thalamic neurons in a well-trained familiar condition.

The Role of the HF in Adaptation to Mismatch Conditions

Various situations inducing sensory mismatch by vection and microgravity in space ships provoke motion sickness with autonomic disturbances in approximately 60% of both healthy subjects and astronauts (Stern et al., 1989). Previous human and animal studies indicated that HF activity was associated with autonomic functions or visceral sensation (Jasper and Rasmussen, 1958; Van Buren, 1963; Pedemonte et al., 2003). Furthermore, the HF sends dense afferent fibers to the autonomic centers including the hypothalamus and amygdala (see a review by Petrovich et al., 2001). These findings suggest that autonomic disturbances in motion sickness might be induced partly by alteration of HF activity induced by novel sensory conflict.

It has been reported that training ameliorates symptoms of motion sickness in subjects susceptible to vection-induced motion sickness (Stern et al., 1989). Furthermore, an artificially generated environment for orientation and motion with similar sensory-conflicts in space is effective for preflight training, which might be mediated through habituation process, and has been used to treat motion sickness (Clemens and Howarth, 2003). Consistent with these clinical studies, repeated exposure to a conflicting condition (backward translocation with locomotion) decreased HF theta activity (Zou et al., 2009). In the present study, some HF place-differential activities encoded sensory information in backward translocation after repeated exposure to this condition. Another study, in which HF place cells were recorded from the rats traversing a 3-dimensional track consisting of the three surfaces during the Neurolab Space Shuttle mission, reported that location-specific firing was initially abnormal or poor, but later became specific to a single surface of the track (Knierim et al., 2000, 2003). These findings suggest that the formation of new place cells encoding new conflicting environmental information might contribute to the updating of stored memory in the HF, enabling a HF comparator to accept this new environment as a match with the stored information.

Furthermore, the present results indicate that the forward-related and backward-related HF activities showed place fields only in specific sessions without any changes in environmental cues. Consistent with this result, cognitive requirements or task paradigms influence place cell activity even in the same environment (Markus et al., 1995; Frank et al., 2000; Wood et al., 2000; Smith and Mizumori, 2006a,b; Takahashi, 2013, 2015). A human behavioral study reported that some subjects were able to adapt context-dependently to different conditions, in which visual information mismatched proprioceptive-vestibular

inputs in a virtual environment (Dumontheil et al., 2006). These findings suggest that the HF is important for context-dependent adaptation.

CONCLUSION

The present results indicate that the HF place cells have the ability to encode a new configuration of sensory inputs, which do not occur naturally, to update information in the HF comparator.

AUTHOR CONTRIBUTIONS

HisN conceived the study. HisN designed the experiment. DZ performed the experiment. DZ and HisN analyzed data and wrote the paper. HisN, HirN, JM, YT, and TO revised the paper. All the authors discussed the results and commented on the manuscript, and read and approved the final manuscript.

FUNDING

The study is supported by research funds from University of Toyama.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2017.00581/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

MINI REVIEW
published: 11 September 2017
doi: 10.3389/fphar.2017.00615

Using the Single Prolonged Stress Model to Examine the Pathophysiology of PTSD

*Rimenez R. Souza[1], Lindsey J. Noble[1,2] and Christa K. McIntyre[2]**

[1] Texas Biomedical Device Center, School of Behavioral and Brain Sciences, University of Texas at Dallas, Richardson, TX, United States, [2] Cognition and Neuroscience Program, School of Behavioral and Brain Sciences, University of Texas at Dallas, Richardson, TX, United States

The endurance of memories of emotionally arousing events serves the adaptive role of minimizing future exposure to danger and reinforcing rewarding behaviors. However, following a traumatic event, a subset of individuals suffers from persistent pathological symptoms such as those seen in posttraumatic stress disorder (PTSD). Despite the availability of pharmacological treatments and evidence-based cognitive behavioral therapy, a considerable number of PTSD patients do not respond to the treatment, or show partial remission and relapse of the symptoms. In controlled laboratory studies, PTSD patients show deficient ability to extinguish conditioned fear. Failure to extinguish learned fear could be responsible for the persistence of PTSD symptoms such as elevated anxiety, arousal, and avoidance. It may also explain the high non-response and dropout rates seen during treatment. Animal models are useful for understanding the pathophysiology of the disorder and the development of new treatments. This review examines studies in a rodent model of PTSD with the goal of identifying behavioral and physiological factors that predispose individuals to PTSD symptoms. Single prolonged stress (SPS) is a frequently used rat model of PTSD that involves exposure to several successive stressors. SPS rats show PTSD-like symptoms, including impaired extinction of conditioned fear. Since its development by the Liberzon lab in 1997, the SPS model has been referred to by more than 200 published papers. Here we consider the findings of these studies and unresolved questions that may be investigated using the model.

Keywords: animal models, fear, glucocorticoids, memory, PTSD, SPS, stress, extinction

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Patrizia Campolongo, Sapienza Università di Roma, Italy
Luigia Trabace, University of Foggia, Italy

***Correspondence:**
Christa K. McIntyre
christa.mcintyre@utdallas.edu

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *08 July 2017*
Accepted: *23 August 2017*
Published: *11 September 2017*

Citation:
Souza RR, Noble LJ and McIntyre CK (2017) Using the Single Prolonged Stress Model to Examine the Pathophysiology of PTSD. Front. Pharmacol. 8:615. doi: 10.3389/fphar.2017.00615

INTRODUCTION

The focus of this *Frontiers in Pharmacology* Research Topic is the neural mechanisms of memory. Memory is a fundamental process in all animals, as it allows survival and success through learned adaptive behaviors. However, some highly stressful experiences can lead to maladaptive fear, anxiety, and protracted periods of suffering like in Posttraumatic Stress Disorder (PTSD). A hallmark symptom of this condition is re-experiencing the traumatic event, suggesting that the problem lies in the mechanisms controlling storage and expression of the traumatic memories. In this mini-review, we will discuss prospective research studies performed in animals to uncover clues about how traumatic experiences can lead to the pathophysiology of PTSD. We also outline some current limitations, knowledge gaps, and areas that require further investigation.

SINGLE-PROLONGED STRESS

Single prolonged stress (SPS) is a frequently used rat model of PTSD. Since its initial description 20 years ago (Liberzon et al., 1997), the SPS procedure has been referred to by over 200 peer reviewed studies. Although it is called a "single" prolonged stress, the procedure is comprised of successive, multi-modal stressors (**Figure 1**). The prolonged stress begins with a 2-h immobilization period that is immediately followed by a forced-swim experience, lasting 20 min, and then a brief loss of consciousness induced by ether exposure. After recovery, rats remain undisturbed for 7 days (Liberzon et al., 1997). In some cases, they are socially isolated (individually housed) during this period (Knox et al., 2012a). When rats undergo auditory or contextual fear conditioning 7 days after this procedure, they demonstrate impaired retention of extinction learning and the conditioned fear response persists longer than it does with fear conditioning alone (Knox et al., 2012a). This approach can be useful for modeling PTSD-like symptoms because those who experience multiple traumas, or a trauma early in life, are more susceptible to developing PTSD following a later traumatic event (Maercker et al., 2004; Anda et al., 2006; Kilpatrick et al., 2013).

Precisely how a previous trauma predisposes individuals to the development of PTSD remains unknown. The first trauma or traumas may simply make an individual more anxious, in general, or more sensitive to future stressors. Alternatively, a previous stressor may set the brain up to acquire, store, or retrieve traumatic memories differently, going forward. Some researchers have hypothesized that an impairment in the recall of fear extinction learning may be an underlying cause of PTSD symptoms (Milad et al., 2008, 2009). The SPS rat model provides an opportunity for testing these hypotheses.

EFFECTS OF SPS ON BEHAVIOR AND THE BRAIN

Many findings suggest that SPS produces behavioral and physiological symptoms that are similar to those observed in PTSD (Liberzon et al., 1997; Kohda et al., 2007; Yamamoto et al., 2010; Knox et al., 2016). Examples of behavioral effects of SPS are illustrated in **Figure 1**. SPS rats demonstrate sleep abnormalities (Vanderheyden et al., 2015) enhanced anxiety (Han et al., 2014; Liu et al., 2016), arousal (Khan and Liberzon, 2004), and fear learning (Iwamoto et al., 2007; Keller et al., 2015b) as well as impaired spatial and recognition memory, social interaction (Kohda et al., 2007; Wen et al., 2016) and fear extinction (Knox et al., 2012a; Keller et al., 2015b). Most changes are observed

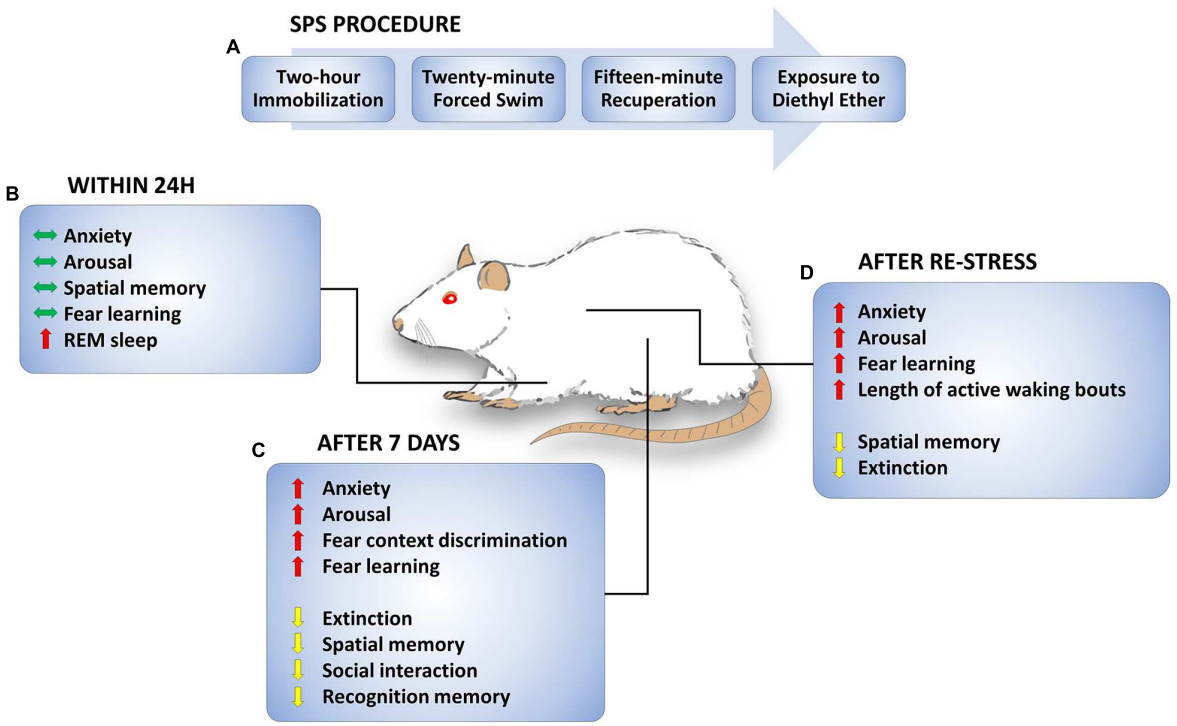

FIGURE 1 | Single prolonged stress (SPS) procedure and SPS-induced behavioral changes. **(A)** Timeline of SPS procedure. On a single day, rats are subjected to a 2-h immobilization followed immediately by a 20-min forced swim. Rats are given a brief period of recuperation and then subjected to diethyl ether until they are anesthetized and unresponsive. **(B)** Behavioral changes observed up to 1 day later. Anxiety, arousal, spatial memory, and fear learning are unchanged. Acute increase in REM sleep and transition to REM sleep is observed. **(C)** Behavioral changes 1 week later. Anxiety, arousal, fear context discrimination, and fear learning are increased. On the other hand, extinction, spatial memory, social interaction, and recognition memory are decreased a week after SPS. **(D)** Behavioral changes following re-stress. Enhanced anxiety, arousal, fear learning, and sleep disturbances remain observed following re-stress, while extinction and spatial memory are impaired. Green, red, and yellow arrows indicate no "changes observed", "increase", and "decrease", respectively.

7 days, but not 1 day, after exposure to the SPS procedure, suggesting that behavioral and cellular changes promoted by SPS are time-dependent (Liberzon et al., 1999a; Knox et al., 2016; Wu et al., 2016). Although it has been demonstrated that partial SPS does not generate extinction impairments (Knox et al., 2012b), the critical features of the SPS procedure for development of a PTSD-like phenotype remain unclear. For example, the passage of time alone may be sufficient for an incubation or sensitization effect following the SPS procedure, or a second stressful experience may be necessary to produce cumulative effects on behavior. In **Figure 1**, behavioral effects of SPS are categorized by the time of testing, i.e., whether testing occurred after SPS, SPS + 7 days (with or without social isolation), or SPS + 7 days + an additional stressor. Though there are variations in some SPS procedures (i.e., social isolation vs. group housing), many studies report consistent SPS effects. For example, social isolation during the quiescent period (Harada et al., 2008) and group housing during the quiescent period (Imanaka et al., 2006) both produced an enhancement in contextual fear conditioning following SPS.

Impaired Extinction of Conditioned Fear

One explanation for the persistence of fear, anxiety, avoidance, and re-experiencing symptoms in PTSD is that some individuals have strong traumatic memories that are less susceptible to extinction. Indeed, some studies of PTSD patients show enhanced conditioned fear (Blechert et al., 2007; Glover et al., 2011; Norrholm et al., 2011), and several animal studies demonstrate an enhancement in contextual fear conditioning following SPS (Iwamoto et al., 2007; Kohda et al., 2007; Keller et al., 2015b). However, others have reported extinction impairments despite normal acquisition of conditioned fear (Milad et al., 2008, 2009; Eskandarian et al., 2013; Vanderheyden et al., 2015; Knox et al., 2016). Using skin conductance responses as a measure of conditioned fear, Milad et al. (2008, 2009) found that PTSD patients showed normal fear conditioning and within-session extinction, but poor retention of extinction on later tests. In rats, prior exposure to the SPS procedure impaired extinction of both cued (Knox et al., 2012a; George et al., 2015; Keller et al., 2015b) and contextual fear conditioning (Yamamoto et al., 2008; Knox et al., 2012a; Matsumoto et al., 2013), whereas acquisition of conditioned fear and extinction within a session were not affected (Knox et al., 2012a,b). Given the evidence that within-session conditioning and retrieval are normal, these findings suggest that consolidation of the extinction memory is impaired in human PTSD patients and in SPS rats. Neurobiological changes that could contribute to impairments in behavior and fear extinction are discussed below (**Table 1**).

Hippocampus

The hippocampus plays a role in storing fear memories and in mediating stress responses (Phillips and LeDoux, 1992; McEwen, 2007). Not surprisingly, the hippocampus is highly sensitive to chronic stress (McEwen, 2007). This is confirmed by functional magnetic resonance imaging (fMRI) studies demonstrating that PTSD patients have a smaller hippocampal volume than healthy controls (Bremner et al., 1995; Stein et al., 1997), although some research suggests that a lower hippocampal volume may represent a risk factor for PTSD (Gilbertson et al., 2002). These findings indicate that reduced hippocampal function might be associated with resistant memory impairments in PTSD.

To our knowledge, no studies have examined the effect of the SPS procedure on hippocampal volume, however, the hippocampus has been the subject of many investigations. Enhanced apoptosis, a phenomenon involved in programed cell death that results in morphological changes, is observed in the hippocampus shortly after SPS, and persists after the undisturbed phase, and after a subsequent stressor (Li et al., 2010; Liu et al., 2010; Wang et al., 2012; Han et al., 2013). Re-stress after SPS also enhances autophagosomes and autophagy-related markers (Wan et al., 2016). Likewise, studies using the SPS model show evidence of enhanced oxidative stress and inflammation (Schiavone et al., 2013). For example, IL-6, malondialdehyde, NOX2, and 4-hydroxynonenal contribute to apoptotic cell death in the hippocampus following SPS (Li et al., 2010; Wang et al., 2012; Han et al., 2013; Liu et al., 2016). Balance and expression of GR and MR receptors is disrupted in the hippocampus of SPS rats. Thus, while decreased expression of GR and MR is observed shortly after SPS (Liberzon et al., 1999a; Zhe et al., 2008), increased expression of these receptors is observed after a week or after re-stress (Zhe et al., 2008; Knox et al., 2012b; Eagle et al., 2013; George et al., 2015). Synaptic plasticity-related mechanisms are also influenced by SPS. Both LTP and LTD are decreased after SPS (Kohda et al., 2007), while NMDA receptor expression is enhanced (Yamamoto et al., 2008). In a recent study using c-Fos expression, Knox et al. (2016) found that SPS disrupted the inhibition of ventral hippocampal activity during extinction retrieval as well as the functional connectivity within the dorsal hippocampus during extinction learning.

Amygdala

The amygdala is also involved in the control of fearful states and learning of emotional experiences. Imaging studies have revealed that PTSD patients show exaggerated amygdala activity in response to trauma-related cues or unrelated arousing stimuli and during new fear learning (Liberzon et al., 1999b; Dunsmoor et al., 2011; Sartory et al., 2013), supporting the notion that enhanced amygdala activity could be involved in impaired extinction learning or generalization of fear responses.

Studies using the SPS model demonstrate changes in the amygdala starting a day after the procedure (**Table 1**). Increased apoptosis and downstream signals, like phosphorylated extracellular signal–regulated kinases, glucose-regulated protein 78 (GRP78) and caspases 3, 9, and 12 expression, were observed in the amygdala 1 day after SPS, and some reached peak levels 7 days later (Liu et al., 2010; Xiao et al., 2011, 2015), suggesting that SPS-induced morphological and connectivity changes may precede the behavioral and memory deficits observed after the 7-day period. Potentiated-fear learning following SPS was paralleled by an early decrease in GR and MR receptors in the amygdala, as well as by blunted LTP and decreased colocalization of GR and MR receptors 1 week later (Kohda et al., 2007; Han

TABLE 1 | Cellular changes in three key areas controlling memory and emotionality after single prolonged stress (SPS) model of PTSD.

Hippocampus	Effect	Reference
Within 24 h	⬆ Apoptosis	Li et al., 2010; Liu et al., 2010; Han et al., 2013
	⇩ Glucocorticoid receptor	Liberzon et al., 1999a; Zhe et al., 2008
	⇩ Mineralocorticoid receptor	Liberzon et al., 1999a; Zhe et al., 2008
	⇩ LTP/LTD*	Kohda et al., 2007
After 7 days	⬆ Apoptosis	Li et al., 2010
	⬆ Glucocorticoid receptor**	Zhe et al., 2008; Knox et al., 2012b; Eagle et al., 2013
	⬆ CB1 receptor	Zer-Aviv and Akirav, 2016
	⬆ NMDA receptor	Yamamoto et al., 2008
	⬆ Oxytocin receptor Binding	Liberzon and Young, 1997
	⬆ IL-6	Liu et al., 2016
	⇩ Glucocorticoid receptor	Ganon-Elazar and Akirav, 2013
	⇩ Mineralocorticoid receptor	Zhe et al., 2008
	⇩ LTP	Kohda et al., 2007
After re-stress	⬆ BDNF	Takei et al., 2011
	⬆ TrkB	Takei et al., 2011
	⬆ Autophagy	Wan et al., 2016
	⬆ Protein kinase M zeta	Ji et al., 2014
	⬆ Apoptosis	Wang et al., 2012
	⬆ Glucocorticoid receptor	George et al., 2015
	⬆ Glycine transporter	Iwamoto et al., 2007
	⬆ Muscarinic receptor	Brand et al., 2008
Amygdala	**Effect**	**Reference**
Within 24 h	⬆ Apoptosis	Xiao et al., 2015
	⬆ pERK	Liu et al., 2010
	⬆ CaM	Xiao et al., 2009
	⇩ Glucocorticoid receptor	Han et al., 2014
	⇩ Mineralocorticoid receptor	Han et al., 2014
After 7 days	⬆ Glucocorticoid receptor	Ganon-Elazar and Akirav, 2013
	⬆ CaM	Xiao et al., 2009
	⬆ Oxytocin	Liberzon and Young, 1997
	⬆ 5-HT2C receptor	Harada et al., 2008
	⬆ Apoptosis	Liu et al., 2010
	⬆ pERK	Liu et al., 2010
	⬆ CB1 receptor	Zer-Aviv and Akirav, 2016
	⬆ Neuropeptide Y	Cui et al., 2008
	⇩ CaMKII	Xiao et al., 2009
After re-stress	⬆ Norepinephrine	Lin et al., 2016a
	⬆ CB1 receptor	Zer-Aviv and Akirav, 2016
	⇩ Dopamine	Lin et al., 2016a
Prefrontal cortex	**Effect**	**Reference**
Within 24 h	⬆ Mineralocorticoid receptor	Zhang et al., 2012
	⬆ CaM	Wen et al., 2012
	⬆ Apoptosis	Li et al., 2013
	⬆ Caspases	Zhang et al., 2016
After 7 days	⬆ Caspases	Wen et al., 2012
	⬆ Glucocorticoid receptor	Knox et al., 2012b; Ganon-Elazar and Akirav, 2013
	⬆ pERK	Wen et al., 2016, 2017
	⬆ CaM	Wen et al., 2012
	⬆ Apoptosis	Wen et al., 2016, 2017
	⇩ Mineralocorticoid receptor	Zhang et al., 2012
	⇩ CaMKII	Wen et al., 2012
	⇩ Glutamate	Knox et al., 2010; Perrine et al., 2016

(Continued)

TABLE 1 | Continued

Prefrontal cortex	Effect	Reference
After re-stress	⬆ Glucocorticoid receptor	Ganon-Elazar and Akirav, 2013; George et al., 2015
	⬆ CB1 receptor	Zer-Aviv and Akirav, 2016
	⇩ CaMKII	Wen et al., 2012
	⇩ Norepinephrine efflux	Lin et al., 2016b
	⇩ Dopamine efflux	Lin et al., 2016b
	⇩ Glutamate	Perrine et al., 2016

*Different from behavioral changes, expression of receptors and other proteins, as well as neurotransmitters, are observed within hours after SPS. Mechanisms controlling neuroendocrine responses, memory and emotion show a full profile of disruption after 7 days of incubation or after subsequent stress experience. CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CB1, cannabinoid receptor 1; pERK, phosphorylated extracellular signal-regulated kinase; BDNF, brain-derived neurotrophic factor; NPY, Neuropeptide Y; LTP/LTD, long-term potentiation/depression; NMDA, ionotropic glutamate receptor; IL-6, Interleukin 6; pPKB, phosphorylated protein kinase B; TrkB, tyrosine receptor kinase B; 5-HT2C receptor, serotonin receptor. *Measured 1 h after SPS; **one study reported decrease.*

et al., 2014). Intracellular calcium levels are changed shortly after SPS and the effect persists for 1 week (Xiao et al., 2009). Acute changes in calmodulin (CaM) and calcium-CaM kinase II (CaMKII), two messengers involved in Ca^{2+} homeostasis and signaling processes related to learning and memory, were up- and downregulated, respectively, within 1 day of SPS (Xiao et al., 2009), indicating that SPS disrupts fundamental mechanisms of cell signaling, which may lead to amygdala hyperactivity, enhanced fear expression and impaired extinction of conditioned fear.

Prefrontal Cortex

Inhibition of amygdala hyperactivity and cognitive flexibility are important prefrontal cortex functions that are implicated in PTSD susceptibility and symptoms (Kitayama et al., 2006; Shin et al., 2006; Gold et al., 2011). This notion is supported by functional imaging studies showing a reduced activity of the medial prefrontal cortex and anterior cingulate cortex in PTSD patients during presentation of trauma-related and non-related aversive stimuli (Shin et al., 2006; Etkin and Wager, 2007; Gold et al., 2011). Moreover, the volume of the ventromedial prefrontal cortex and the anterior cingulate cortex is reduced in individuals with PTSD (Kitayama et al., 2006; Kasai et al., 2008; Karl and Werner, 2010). Abnormal morphological changes in the pathway from the anterior cingulate cortex to the amygdala was also found in PTSD patients (Kim et al., 2006), suggesting that a series of changes in the normal control of the fearful states or behavioral flexibility by the frontal cortex may be involved in the pathophysiology of PTSD.

Evidence for similar changes in the prefrontal cortex of rats submitted to the SPS model remains sparse. As in the hippocampus and amygdala, neuronal apoptosis and dysregulation of autophagic activity in the prefrontal cortex appears 1 day after SPS (Li et al., 2013; Wen et al., 2016; Zheng et al., 2017). Elevated levels of protein kinase RNA-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) in the endoplasmic reticulum (ER), glucose-regulated protein (GRP) 94 and apoptosis-related caspase-12 are involved in the persistent apoptotic profile seen 1 week after SPS (Li et al., 2013; Zhao et al., 2014; Wen et al., 2016, 2017). Unbalanced control of calcium indicates that intracellular messengers controlling neuronal excitability are disrupted following SPS (Wen et al., 2012). This is corroborated by studies showing decreased levels of glutamate in the prefrontal cortex 1 week after SPS or re-stress (Knox et al., 2010; Perrine et al., 2016). The concentration of MRs is elevated 1 day after SPS (Zhang et al., 2012), while GR expression is enhanced 1 week later and after re-stress (Knox et al., 2012b; Ganon-Elazar and Akirav, 2013; George et al., 2015), indicating temporally distinct disturbances in stress-related systems.

Decreased volume and integrity of prefrontal sub-regions have been reported in PTSD patients (Rauch et al., 2003; Woodward et al., 2006). Similarly, SPS disrupts normal activity of the infralimbic region of the medial prefrontal cortex before re-stress (Knox et al., 2016), suggesting that SPS could predispose the prefrontal cortex to dysfunctional activity during fear learning and/or subsequent extinction trials. However, since different regions of the prefrontal cortex control distinct aspects of fear learning and extinction, additional studies are needed for a better understanding about changes that can be predisposing factors or consequences of the trauma.

Effects of SPS on HPA-Axis

Early research on the pathophysiology of PTSD identified a decrease in cortisol levels (Yehuda et al., 1990). Later studies demonstrated that administration of low doses of dexamethasone produced suppression of plasma cortisol, indicating that the hypothalamus-pituitary-adrenal cortex (HPA) axis may become sensitive to negative feedback in PTSD patients (Yehuda et al., 1995). Similarly, enhanced suppression of the HPA-axis is observed in rats 7 days after SPS (Liberzon et al., 1997, 1999a). The data currently available suggest that the enhanced glucocorticoid negative feedback observed in SPS may be linked to overexpression of GR and a reduced expression of MR in key areas mediating activity of the HPA-axis during stress (Liberzon et al., 1999a; Zhe et al., 2008; Eagle et al., 2013).

Changes in the HPA-axis may contribute to PTSD symptoms by interfering with extinction of conditioned fear. For example, exogenous administration of stress-levels of cortisol can impair the retrieval of long-term memories (de Quervain et al., 1998), but the same treatment enhances consolidation of new memories (McGaugh and Roozendaal, 2002; de Quervain et al., 2009). These findings suggest that SPS-induced enhanced suppression of the HPA-axis may have the opposite effect, perpetuating the

fear memory by facilitating retrieval of the traumatic memory and impairing consolidation of extinction memory (de Quervain et al., 2017). However, a few studies have dissociated GR upregulation and extinction impairments in the SPS model. A significant increase in GR expression was observed in the hippocampus and prefrontal cortex 7 days after partial SPS (e.g., forced swimming and ether exposure) that did not impair extinction of conditioned fear (Knox et al., 2012b). These results indicate that glucocorticoid receptor expression must reach a threshold in order to interfere with the consolidation of extinction, or there is another SPS-related change that influences the extinction of conditioned fear. Consistent with the view that enhanced suppression of the HPA-axis and the resulting decrease in circulating glucocorticoids predisposes animals to the PTSD phenotype, Keller et al. (2015b) found that inhibition of corticosterone synthesis prior to fear conditioning exacerbated the extinction impairment in SPS rats.

Taken together, these findings indicate that the SPS model is a useful tool for studying the role of the HPA-axis in PTSD. Future studies should examine the full extent of HPA-axis changes, including the evaluation of SPS effects on circulating glucocorticoid levels. Further studies may be designed to determine whether HPA-axis dysfunction is a predisposing factor or a consequence of traumatic experience.

LIMITATIONS

Many PTSD-like effects have been identified in rats exposed to SPS. However, seemingly subtle deviations in the procedure may have significant consequences on behavior and physiology (Knox et al., 2012b). In this review, we have sorted the behavioral and physiological consequences of SPS by the time of testing. Some effects are transient, and some emerge after 7 days or a re-stress experience, suggesting that the effects of SPS are time- and experience-dependent. Variations on SPS parameters can be utilized to identify factors producing maladaptive fear and arousal states. Future studies are needed to determine the relative contributions of the passage of time and stress experience to these SPS-related changes.

Here, we also describe evidence that extinction impairments are a common feature of PTSD and the rat SPS model of PTSD. A major caveat is that human females are two times more likely to develop PTSD following a traumatic event (Kessler et al., 2005), yet SPS-induced deficits in extinction are only seen in male rats. In one study that investigated sex differences in the SPS model, Keller et al. (2015a) demonstrated that SPS affects GR expression in the dorsal hippocampus in females, but extinction retention deficits were observed only in males, suggesting that female rats are more resilient to the memory extinction effects of SPS. Such differences may be indicative of a sexually divergent response to conditioned fear. Emerging evidence indicates that female rats express fear by darting rather than freezing (Gruene et al., 2015), indicating that reliance on freezing as a single measure of fear may be misleading.

CONCLUSION

Although we have focused on factors contributing to extinction impairments, the SPS model can be used to investigate hypotheses about the biological causes of other debilitating symptoms such as social withdrawal, heightened anxiety, elevated startle response, hypervigilance, and sleep disturbances. Though the SPS model is a useful tool to study the PTSD symptomatology, additional studies are needed to examine sex differences, the timing of onset and persistence of symptoms, as well as the features of the SPS procedure that are necessary for the development of PTSD-like symptoms. Given the understanding that all models have limitations, it is encouraging to note that several other animal models demonstrate extinction impairments and PTSD-like symptoms (Izquierdo et al., 2006; Matsumoto et al., 2008; Wilber et al., 2009; Goswami et al., 2010; Long and Fanselow, 2012). Utilization of multiple animal models of PTSD and meticulous examination of PTSD-like symptoms will be critical to unfold the pathophysiology of PTSD, and lead to novel and efficient therapeutic strategies.

AUTHOR CONTRIBUTIONS

All authors have been studying the Single Prolonged Stress (SPS) rat model of PTSD in the lab for over 1 year. LN and CM met to discuss writing a mini review on the subject of SPS effects on the brain that may be responsible for the PTSD-like symptoms that we and others have observed in this model. We came up with an outline and invited RS to contribute a summary of physiological effects of SPS. LN wrote about behavioral effects. CM combined both portions, edited, and added some discussion. RS produced the figure and table.

FUNDING

This work was sponsored by the Defense Advanced Research Projects Agency (DARPA) Biological Technologies Office (BTO) Electrical Prescriptions (ElectRx) program under the auspices of Dr. Doug Weber through the Space and Naval Warfare Systems Center, Pacific. Grant/Contract No. DARPA-BAA-14-38 and DARPA-BAA-15-06 and by the NIMH, MH105014.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 12 September 2017
doi: 10.3389/fphar.2017.00621

Stress-Induced Reduction of Dorsal Striatal D2 Dopamine Receptors Prevents Retention of a Newly Acquired Adaptive Coping Strategy

*Paolo Campus[1,2], Sonia Canterini[1], Cristina Orsini[1,3], Maria Teresa Fiorenza[1,3], Stefano Puglisi-Allegra[1,3] and Simona Cabib[1,3]**

[1] Department of Psychology, Center 'Daniel Bovet', Sapienza Università di Roma, Rome, Italy, [2] Department of Psychiatry, University of Michigan, Ann Arbor, MI, United States, [3] Fondazione Santa Lucia (IRCCS), Rome, Italy

OPEN ACCESS

Edited by:
Antonella Gasbarri,
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Reviewed by:
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Gina Lorena Quirarte,
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***Correspondence:**
Simona Cabib
simona.cabib@uniroma1.it

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 02 July 2017
Accepted: 24 August 2017
Published: 12 September 2017

Citation:
Campus P, Canterini S, Orsini C, Fiorenza MT, Puglisi-Allegra S and Cabib S (2017) Stress-Induced Reduction of Dorsal Striatal D2 Dopamine Receptors Prevents Retention of a Newly Acquired Adaptive Coping Strategy. Front. Pharmacol. 8:621. doi: 10.3389/fphar.2017.00621

The inability to learn an adaptive coping strategy in a novel stressful condition leads to dysfunctional stress coping, a marker of mental disturbances. This study tested the involvement of dorsal striatal dopamine receptors in the dysfunctional coping with the Forced Swim test fostered by a previous experience of reduced food availability. Adult male mice were submitted to a temporary (12 days) reduction of food availability [food-restricted (FR)] or continuously free-fed (FF). Different groups of FF and FR mice were used to evaluate: (1) dorsal striatal mRNA levels of the two isoforms of the dopamine D2 receptor (D2S, D2L). (2) Forced Swim-induced c-fos expression in the dorsal striatum; (3) acquisition and 24 h retention of passive coping with Forced Swim. Additional groups of FF mice were tested for 24 h retention of passive coping acquired during a first experience with Forced Swim immediately followed by intra-striatal infusion of vehicle or two doses of the dopamine D2/D3 receptors antagonist sulpiride or the D1/D5 receptors antagonist SCH23390. Previous restricted feeding selectively reduced mRNA levels of both D2 isoforms and abolished Forced Swim-induced c-fos expression in the left Dorsolateral Striatum and selectively prevented 24 h retention of the coping strategy acquired in a first experience of Forced Swim. Finally, temporary blockade of left Dorsolateral Striatum D2/D3 receptors immediately following the first Forced Swim experience selectively reproduced the behavioral effect of restricted feeding in FF mice. In conclusion, the present results demonstrate that mice previously exposed to a temporary reduction of food availability show low striatal D2 receptors, a known marker of addiction-associated aberrant neuroplasticity, as well as liability to relapse into maladaptive stress coping strategies. Moreover, they offer strong support to a causal relationship between reduction of D2 receptors in the left Dorsolateral Striatum and impaired consolidation of newly acquired adaptive coping.

Keywords: dopamine receptors, dorsolateral striatum, helplessness behavior, hemispheric bias, memory consolidation, sustained threat

INTRODUCTION

The ability to respond to stressors with adaptive coping strategies is determinant for the psychological well-being of human and non-human animals (Koolhaas et al., 2010; Maier and Watkins, 2010; Cabib and Puglisi-Allegra, 2012; Helmreich et al., 2012; Pomerantz et al., 2012; Andolina et al., 2013; de Kloet and Molendijk, 2016). Indeed, dysfunctional stress coping characterizes different mental diseases (Taylor and Stanton, 2007; Aldao and Nolen-Hoeksema, 2010; Moritz et al., 2016). Therefore, animal models of stress coping have major translational value for research.

Although immobility expressed by rodents in the Forced Swim test (FSt) has been used as a measure of depressive-like behavior, there is a large consensus on the view that this behavioral response is an adaptive strategy to cope with a stressful situation that cannot be avoided nor escaped (Cabib et al., 2012; Andolina et al., 2013; Campus et al., 2015; de Kloet and Molendijk, 2016). During their first FSt experience (10 min for mice 15 min for rats) animals show initial expression of vigorous active coping (swimming around and struggling to climb the container's walls). These responses decrease overtime whereas episodes of immobility (only small movements required to keep the head above water) increase in frequency and duration. The immobility response prevents useless and risky loss of energy, thus it is acquired and consolidated as long-term memory to be immediately adopted on subsequent encounters with the stressor (Mitchell and Meaney, 1991; Colelli et al., 2014; Reul, 2014).

Development or expression of immobility in FSt is disrupted by proximal stress experiences (Molina et al., 1994; Alcaro et al., 2002; Becker et al., 2008; Mozhui et al., 2010), an observation that further support the translational value of this animal model because proximal adverse experiences contribute to development of mental diseases (Dias-Ferreira et al., 2009; Daskalakis et al., 2013; Gourley et al., 2013; Diwadkar et al., 2014; Reul, 2014; Eagle et al., 2015). The neurobiological mechanisms mediating the disruptive effects of proximal stress experiences on subsequent coping with FSt are still poorly understood, although disturbances of learning processes could play a major role (Mitchell and Meaney, 1991; Colelli et al., 2014; Reul, 2014).

In the present study we tested the involvement of dorsal striatal D2 dopamine receptors (D2R) in impaired consolidation of newly acquired passive coping response to FSt fostered by a temporary reduction of food availability. Indeed, strong evidence supports a role for the dorsolateral striatum in consolidation of a long-term memory of immobility in FSt (Colelli et al., 2014; Campus et al., 2015) and recent findings offer support to the involvement of impaired memory consolidation in stress-induced disruption of FSt coping in mice (Campus et al., 2016). Moreover, different types of stressors have been shown to alter availability of D2R in the mouse brain (Puglisi-Allegra et al., 1991; Cabib et al., 1998; Patrono et al., 2015) and D2R play a major role in memory consolidation (Sigala et al., 1997; Setlow and McGaugh, 1999; Manago et al., 2009; Lee and Chirwa, 2015). Finally, unseasonal reduction of food availability represents an unpredictable stressor in natural settings (Wingfield and Kitaysky, 2002), thus it models an ecologically meaningful stressor in laboratory settings.

MATERIALS AND METHODS

Animals and Housing

Male mice of the inbred DBA/2J strain (Charles River, Como, Italy) were purchased at 6 weeks of age and housed in groups of four in standard breeding cages with food and water *ad libitum* on a 12-h dark/light cycle (lights on between 07:00 and 19:00 h) at a temperature of $22 \pm 1°C$.

At 7 weeks of age mice were all individually housed and assigned to free-feeding (FF) or food-restricted (FR) condition. FF mice received food once daily in a quantity adjusted to exceed daily consumption (15 g). FR mice received food once daily in a quantity adjusted to lose 15% of the initial body weight within the first 3 days and maintain the reached weight for the following 9 days. On the evening of the 12th day of individual housing mice from both FF and FR groups were given food *ad libitum* and left undisturbed for 48 h before behavioral testing or tissue collection.

All animals were treated humanely in accordance with the principles expressed in the Declaration of Helsinki. Experimental protocols and related procedures were approved by Italian Ministry of Public Health. All efforts were made to minimize animal suffering, according to European Directive 2010/63/EU on the use of animals for research.

RNA Isolation and Gene Expression Analysis by Quantitative Real-time RT-PCR

Mice were killed by cervical dislocation and then decapitated for brains excision. Brain was quickly removed, frozen in dry ice and sliced with a freezing microtome. A stainless steel needle (1 mm inside diameter) was used to punch DLS and DMS from coronal slices no thicker than 300 μm (three slices from 1.05 to 0.15 from bregma, MBL Mouse Brain Atlas DBA/2[1]). Locations of punches are shown in **Figure 1**.

Punches were processed for total RNA extraction using the RNeasy Mini kit (Qiagen). To reduce variability in the total RNA yields, the automated QiaCube instrument (Qiagen, Hilden, Germany) was used to purify RNAs. Purified RNA samples were treated with DNase I to remove genomic DNA and cDNA was synthesized using Superscript III reverse transcriptase (Life Technologies, Rockville, MD, United States), acc.to the protocol supplied by the manufacturer.

Real-time RT-PCR was performed on a EcoTM Illumina thermal cycle using QuantiFast Sybr Green PCR Master Mix (Qiagen, Inc., Valencia, CA, United States). Amplification reaction conditions were as follows: 95°C for 10 min, then 40 cycles of 95°C for 15 s and 58°C for 60 s.

Primer sequences were: D2L_forward: 5′-AACTGTACCC ACCCTGAGGA-3′; D2S_forward 5′-CACCACTCAAGGATGC TGCCCG-3′; D2_reverse: 5′-GTTGCTATGTAGACCGTG-3′;

[1]http://www.mbl.org/atlas165/atlas165_start.html

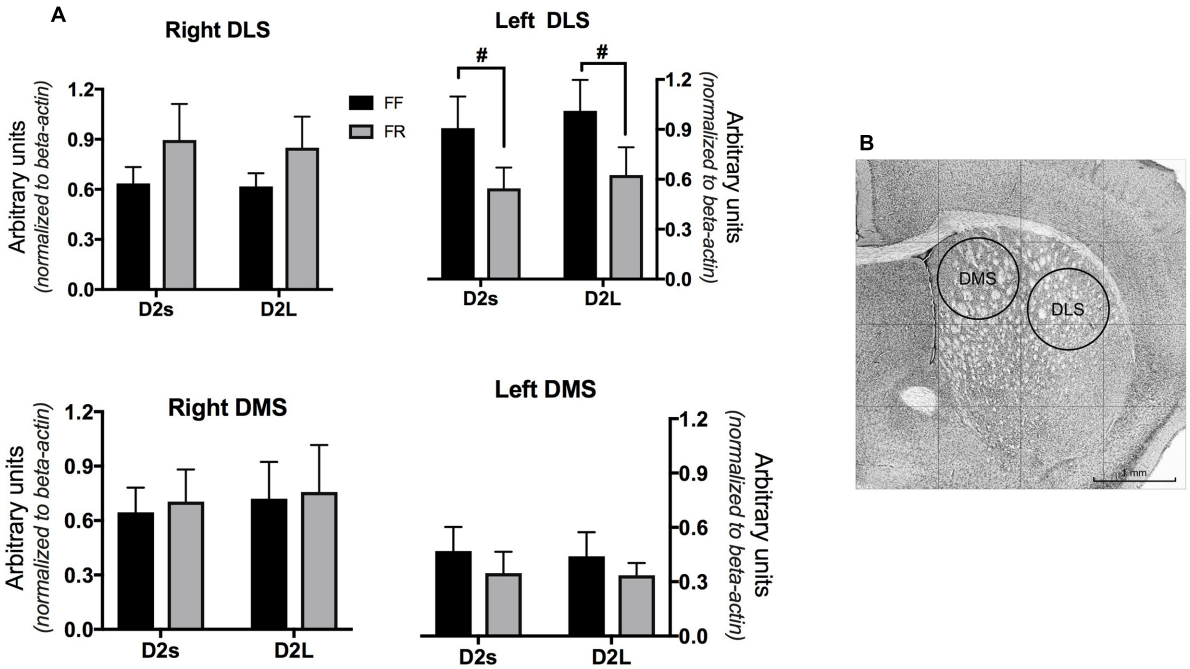

FIGURE 1 | (A) mRNA level of the two isoforms of D2 dopamine receptors (short D2S; long D2L) in the dorsomedial and dorsolateral striatum of continuously free fed (FF) and temporarily food restricted (FF) mice. Data are expressed as mean arbitrary units ± SEM. **(B)** Punches location and dimension (example from the left hemisphere). #Main effect of the feeding condition (see Results).

β′actin_forward 5′-GAAATCGTGCGTGACATCAAAG-3′; β′actin_reverse 5′-TGTAGTTTCATGGATGCCACAG-3′. The size of amplified PCR product was 228 bp (D2L), 155 bp (D2S), and 216 bp (beta-actin).

The amplification efficiency for each primer pair was preliminarily determined by amplifying serial dilution (0.32–200 ng) of total striatum cDNA obtaining a linear standard curve. All primer pairs showed good linearity and similar amplification efficiency (98–99%) with all primers pairs. To control for products specificity, a melting temperature dissociation curve analysis was performed, from 55 to 95°C, measuring fluorescence every 0.5°C and the amplified PCR products were visualized after electrophoresis on 1.8% agarose gel. Each experiment was performed in triplicate and values were averaged. For each primer pairs, negative controls without cDNA were included to rule out genomic DNA contamination. Beta actin expression was found to be unaffected by our experimental design in all brain regions examined, and was therefore used as the internal control for normalization. Similar results were obtained when GAPDH was used for normalization.

Forced Swimming Test

Apparatus and procedures were previously described (Colelli et al., 2014; Campus et al., 2015). Briefly, the procedure consisted of a first and a second experience separated by 24 h time interval. Each mouse was gently laid in the water contained in a glass cylinder and left for 10 min on the first experience (training) and 5 min on the second (test). Both sessions were registered by a digital video camera located frontally to the apparatus and connected to a computer located within a different room.

Duration (sec) of struggling to climb out, swimming, and immobility (absence of all movements not required to float), was scored on videotapes by a trained experimenter, unaware of the experimental groups each animal belonged to, using EthoVision (Noldus Netherlands).

Immunohistochemistry

Mice were killed by cervical dislocation and then decapitated for brains excision. Brains were removed, post-fixed, and cryoprotected as described previously (Conversi et al., 2006; Colelli et al., 2010). Tissue was sliced on the coronal plane into 40 μm thick sections. Rabbit anti- c-fos (1/20000; Oncogene Sciences) was used as primary antibody. Peroxidase staining was obtained by standard avidin–biotin procedure using the rabbit Vectastain Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA, United States) and chromogenic reaction was developed by incubating sections with metal-enhanced DAB (Vector Laboratories, Inc., Burlingame, CA, United States), according to the protocol supplied by the manufacturer. Images of dorsolateral (DLS) and dorsomedial (DMS) striatum were acquired with a Nikon Eclipse 80i microscope equipped with a Nikon DS-5M CCD camera. The analysis of images was performed by using the public domain image analysis software IMAGEJ 1.48 (Abramoff et al., 2004). Immunoreactive nuclei quantification was expressed as density (number of nuclei/0.1 mm^2).

Temporary Inactivation of Dopamine Receptors in the Dorsolateral Striatum

After 1 week of individual housing, continuously free-fed (FF) mice were implanted with stainless-steel guide cannulas unilaterally in the left dorsolateral striatum (DLS) as previously described (Colelli et al., 2014; Campus et al., 2015). Briefly, mice were anesthetized with Zoletil 100, Virbac, Milano, Italy (tiletamine HCl 50 mg/ml+zolazepam HCl 50 mg/ml) and Rompun 20, Bayer S.p.A Milano, Italy (xylazine 20 mg/ml) purchased commercially, and mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, United States). A single stainless-steel guide cannula (Unimed, Geneva, Switzerland: 7 mm in length, 0.50 mm in external diameter) was inserted in the left DLS (+0.8 mm anterior to bregma, ± 2.3 mm lateral to midline, −2.5 mm ventral from the skull according to Franklin and Paxinos, 2001). One week later, drugs were infused (total volume 0.4 μl, flow rate of 0.2 μl/min) through a stainless-steel injection cannula (Unimed, Geneva, Switzerland, 0.11 mm in internal diameter) connected by polyethylene catheter tubing to a 1 μl Hamilton micro-syringes (Hamilton, Co., Reno, NV, United States), as previously described (Colelli et al., 2014; Campus et al., 2015). Infusions were performed immediately after the firs FSt experience and behavioral test was performed 24 h later.

Doses of dopamine agonists were chosen in preliminary experiments. The D1 dopamine receptor antagonist SCH 23390 (Sigma Aldrich) was dissolved in a 0.90% saline solution and infused at one of two doses (0.5 or 1 μg/mouse); the D2/D3 dopamine receptor antagonist sulpiride (Sigma Aldrich) was first dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted in 0.90% saline up to reaching two different final concentrations (0.5 or 1 μg/mouse). The final DMSO concentration never exceeded 10%; 0.90% saline or 0.90% saline +10% DMSO were infused in control (0) groups.

Experimental Protocols and Statistics

Because of previous evidences for the selective involvement of the left DLS in consolidation of a long-term memory of passive coping acquired in FSt (Colelli et al., 2014; Campus et al., 2015), we separately evaluated D2R mRNA levels and c-fos immunostaining in the left and right hemispheres and statistically tested a possible bias by including the factor 'Hemisphere' in the ANOVAs.

All FR mice were behaviorally tested or sacrificed after 12 days of restricted feeding followed by 48 h of free food availability (14 days of individual housing). All FF mice were behaviorally tested or sacrificed following 14 days of individual housing.

A total of eight groups of FR and of 14 groups of FF mice were used for the present experiment.

One group of FF and one group of FR mice ($n = 6$ each) were used to quantify D2R mRNA levels. D2R mRNA determinations obtained from triplicate experiments were averaged for each subject and statistically analyzed by three-way ANOVAs (Isoform = two levels: D2L, D2S; Feeding = two levels: FF, FR and Hemisphere = two levels: Left, Right). Additionally, we evaluated a possible difference in hemispheric availability of the two D2R isoforms in FF and FR mice separately, by two-way ANOVAs (Isoform × Hemisphere).

Two groups of FF and two groups of FR mice were used for c-fos immunostaining experiments. Six mice from each feeding condition were sacrificed immediately after removal from their home cages (naïve). Six mice from each feeding condition were sacrificed 50 min after a first experience of forced swim (10 min, FSt). Statistical analyses of c-fos data were performed on number of immunostained nuclei/0.1 mm^2 from each sampled area. Three-way ANOVAs were used (two between factors: Experience of forced swim = two levels: Naïve, Experienced; Feeding = two levels: FF, FR; and one within factor: Hemisphere = two levels: Left, Right). When allowed by the results, individual between-groups comparisons were performed *post hoc* (Sidak correction).

A group of FF ($n = 6$) and a group of FR ($n = 6$) mice were used to evaluate the effects of a previous experience of restricted feeding on behavioral responses to FSt. Statistical analyses were performed on duration (sec) of the different behaviors expressed in the first and second block of 5 min of the first FSt experience and on the 5 min test performed 24 h later. A two-way ANOVAs for repeated measures (min blocks, three levels: 0–5, 6–10 min, test) with Feeding as between factor was employed. When allowed by results obtained, significant difference with behavior expressed during the first 5 min of exposure to FSt was tested *post hoc* (Paired *t*-test).

Six groups (one group for vehicle and one for each of the two doses of each antagonist) of FF mice ($n = 7$ each) were used to evaluate the effects of immediate post-experience infusion of DA antagonist in the left DLS on consolidation of a passive coping response (immobility). All mice were exposed to 10 min of forced swimming on the 14th day of individual housing and tested the following day. Immobility duration (sec) expressed on the final test was statistically analyzed by a two-way ANOVA for independent measures (Feeding, two levels = FF, FR; Dose, three levels = 0, 0.5, 1 μg/mouse). When allowed, individual between-groups comparisons were performed *post hoc* (Sidak correction).

RESULTS

Restricted Feeding Selectively Reduced D2R mRNA Level in the Left Dorsolateral Striatum

Figure 1 shows data on dorsal striatal D2L and D2S mRNA levels indicating a selective decrease of both D2R isoform limitedly to the left dorsolateral striatum (DLS) of FR mice.

Indeed, statistical analyses only revealed a significant interaction between the three factors (Feeding × Hemisphere × Isoform) in the DLS [$F(1,40) = 7.307$; $p < 0.05$] due to significant [$F(1,10) = 7.29$; $p < 0.05$] reduction of mRNA levels of both isoforms in the FR group (**Figure 1**).

Because larger D2R availability has been observed in the left striatum of rats and healthy humans (Schneider et al., 1982; Tomer et al., 2008), we separately compared hemispheric levels of the two D2R isoforms in the different striatal compartment of

FF and FR mice. A significant hemispheric difference was found in the DLS of FF mice only, due to a left bias for both isoforms [$F(1,20) = 5.136$; $p < 0.05$].

Restricted Feeding Selectively Prevented Forced Swim-Induced c-fos Expression in the Left Dorsolateral Striatum

In **Figure 2** are shown data on induction of c-fos immunostaining by a first FSt (10 min) experience in the right and left striatum of FF and FR mice. These data indicate a selective induction of c-fos expression in the left DLS of FF mice that was absent in FR mice.

Thus, statistical analyses revealed a significant main effect of FSt experience in the dorsomedial striatum (DMS) [$F(1,40) = 38.36$; $p < 0.0001$] due to a significant increase of c-fos immunostaining in FSt-experienced mice regardless of the hemisphere or of the feeding condition. A significant global interaction was revealed for c-fos expression in the DLS [$F(1,40) = 4.182$; $p < 0.05$] due to a significant c-fos expression in the left DLS of FF mice only (**Figure 2**).

Either Restricted Feeding or Pharmacological Inactivation of D2/D3 Receptors in the Left Dorsolateral Striatum Prevented 24 h Retention of the Passive Coping Strategy Acquired in the First Experience with Forced Swim Test

Data on behavior expressed in FSt are presented in **Figure 3**; they indicate that whereas both FF and FR mice developed a passive coping strategy in the course of the first experience with FSt only FF mice retained this strategy for the following 24 h. Moreover, they indicate that pharmacological blockade of D2/D3R in the left DLS immediately after the first FSt experience reproduces the behavioral effects of restricted feeding in FF mice.

Figure 3A shows mean duration of the different behaviors expressed during the first (two blocks: 0–5, 6–10 min) and second (test: 5 min) experience of FSt by FF and FR mice. Statistical analyses revealed a significant interaction between factors (Feeding × Repeated measure) for both immobility: $F(2,20) = 4.251$; $p < 0.05$, and Swim: $F(2,20) = 5.541$; $p < 0.05$. *Post hoc* comparisons (paired *t*-test) revealed a significant increase of immobility and a significant reduction of swimming between the first and second 5 min of the first FSt experience regardless of the feeding condition. Levels of immobility and swimming were still significantly different from those expressed during the first 5 min of FSt experience in FF mice tested 24 h later but not in FR mice (**Figure 3A**).

In **Figure 3B** are reported data on the effects of immediately post-FSt unilateral infusion of SCH23390 (D1 antagonist) or Sulpiride (D2/D3 antagonist) in the left DLS on immobility expressed on the retrieval test performed 24 h later. Statistical analyses did not reveal any significant effect of the D1R antagonist whereas infusion of the D2/D3 antagonist dose-dependently reduced the amount of immobility expressed on the retrieval test [$F(2,17) = 4.45$; $p < 0.05$].

DISCUSSION

Major findings of the present study are: (1) the decrease of D2R mRNA in the left DLS of FR mice; (2) the selective inhibition of FSt-induced c-fos expression in the left DLS of FR mice; (3) the shared ability of pharmacological blockade of D2/D3R in the left DLS and of previous experience of reduced food availability to prevent 24 h retention of FSt-induced immobility.

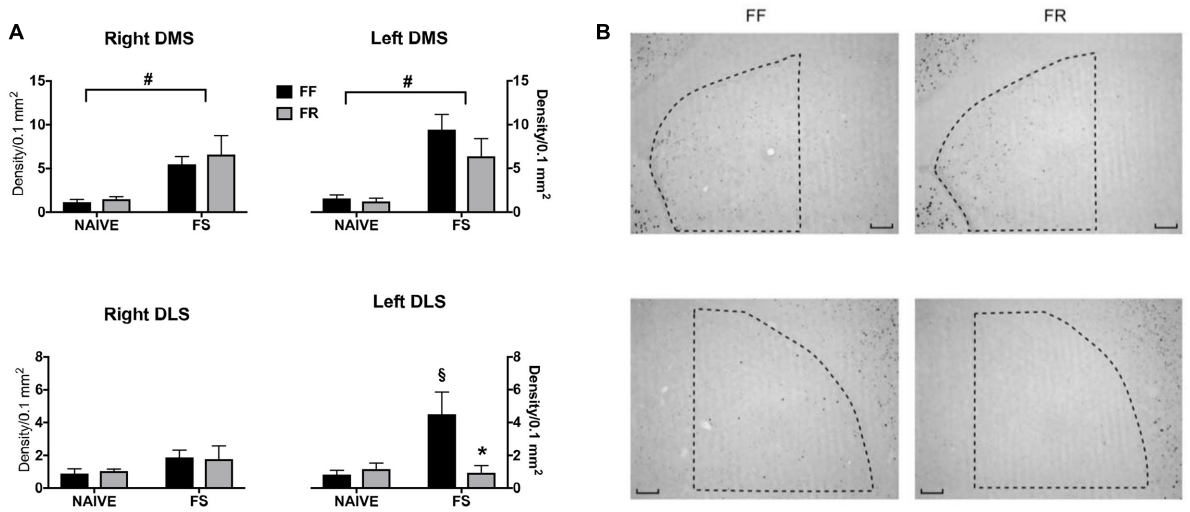

FIGURE 2 | Effects of 12 days or restricted feeding on c-fos immunostaining promoted by a first experience (10 min) of Forced Swim. **(A)** Quantitative data (mean density/0.1 mm^2 ± SEM). #Main effect of the Forced Swimming experience (see Results). §$p < 0.005$ vs. Naive (not exposed to FS) (Sidak correction for multiple comparisons). *$p < 0.05$ vs. continuously free-fed (FF) mice exposed to Forced Swim (Sidak correction for multiple comparisons). **(B)** Representative staining of samples from the left DMS (top) and left DLS of continuously free-fed (FF) and temporarily food-restricted (FR) mice exposed to 10 min of forced swimming.

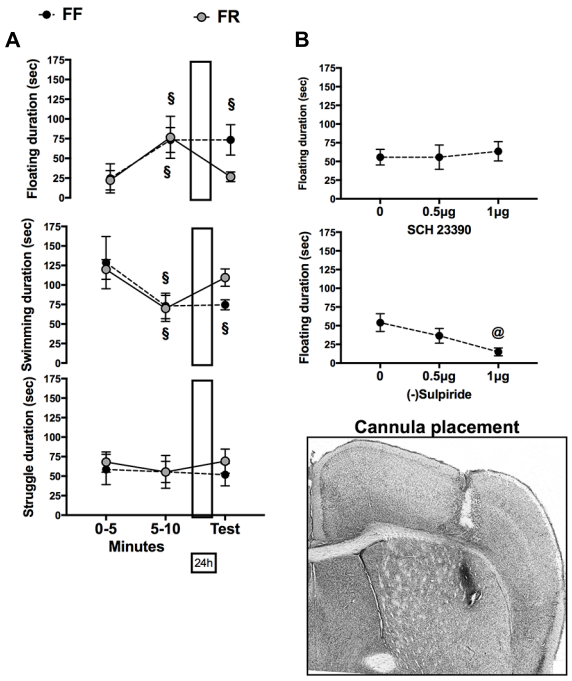

FIGURE 3 | (A) Effects of restricted feeding on acquisition (0–5, 5–10 min) and retrieval (Test) of immobility (top), swimming (middle), and struggling (bottom) behavior. **(B)** Effects of immediate post-training infusion of the D1 receptor antagonist SCH 23390 (top) or the D2 receptor antagonist sulpiride (bottom) in the left DLS on immobility expressed on the retention test performed 24 h later. Data are expressed as mean duration (±SEM) of behavior. $^{\S}p < 0.05$ vs. behavior expressed during the first 5 min of FS experience (0–5) (paired *t*-test). $^{@}p < 0.05$ vs. vehicle infusion (0) (Sidak correction for multiple comparisons).

In a first set of experiments we observed a selective reduction of D2R mRNA levels in the left DLS of FR mice. To the best of our knowledge this is the first report of a reduction of striatal D2R availability following exposure to adverse environmental conditions in mice. Indeed, previous studies using different chronic/repeated stress protocols and different measures of receptors availability reported an increase of D2R in the ventral striatum and no significant changes in the dorsal striatum of stressed mice (Cabib et al., 1988, 1998; Lim et al., 2011; Patrono et al., 2015).

Reduced striatal D2R availability is considered a main marker of addiction-associated aberrant plasticity because it has been reported in human addicts (Martinez et al., 2004; Tomasi and Volkow, 2013), in monkeys after chronic escalating methamphetamine (Groman et al., 2012) or prolonged cocaine self-administration (Nader et al., 2006), and in rats following cocaine self-administration (Besson et al., 2013). Thus, the finding of the present study is coherent with the ability of restricted feeding to produce phenotypes fostered by prolonged exposure to addictive drugs in laboratory animals (Carr, 2011; Zheng et al., 2012; Branch et al., 2013). Indeed, mice exposed to the protocol used for the present experiments show behavioral markers of addiction-associated aberrant neuroplasticity (Cabib et al., 2000).

Because of previous observations that the splicing of the D2R gene might influence addiction liability in humans (Moyer et al., 2011) and is influenced by exposure to addictive drugs in mice (Giordano et al., 2006), we measured the relative abundance of the two D2R isoforms. Our finding that both D2L and D2S undergo a similar decrease of their expression level in the left DLS of FR mice do not support an effect of restricted feeding on D2R gene splicing, pinpointing a possible modulation of the transcriptional activation of this gene.

Finally, the present study revealed that FR-induced decrease of D2R mRNA was confined to the left DLS. This finding could explain previous failure to demonstrate significant effects of stress on DLS D2R availability (Cabib et al., 1988, 1998; Lim et al., 2011; Patrono et al., 2015). Moreover, it is in line with evidences of lateralized brain stress responses (Berridge et al., 1999; Sullivan and Dufresne, 2006; Cerqueira et al., 2008; Lupinsky et al., 2010) and with lateralization of mesostriatal DA transmission (Schneider et al., 1982; Molochnikov and Cohen, 2014). To this regard, it is worth pointing out that higher levels of D2R have been observed in the left striatum in both healthy humans and rats (Schneider et al., 1982; Tomer et al., 2008), a phenomenon confirmed by results of the present experiments in the DLS of FF but not FR mice.

Because of our previous finding of a main role of the left DLS in consolidation of immobility acquired in FSt (Colelli et al., 2014), the selective reduction of D2R in the left DLS of FR mice was strongly suggestive of an alteration of the memory processes engaged by FSt in these mice. Therefore, we evaluated the effects of a previous FR experience on development and 24 h retention of FSt-induced passive coping. The results obtained indicated a selective effect of restricted feeding on retention rather then on expression or acquisition of the coping strategy (**Figure 3A**), supporting a role for altered memory processing in the impaired coping with FSt observed in FR mice.

The experience of restricted feeding also abolished FSt-induced c-fos expression in the left DLS (**Figure 2**). Indeed, in line with previous findings (Colelli et al., 2014), FF mice responded to a first 10 min-long FSt experience with increased c-fos expression in the left DLS a response that was not observable in FR mice. The effect of restricted feeding on c-fos expression induced by a first experience of forced swim support the hypothesis of altered consolidation of a newly acquired passive coping strategy. Indeed, expression of c-fos has been associated and causally related with consolidation of long-term memories (Lamprecht and Dudai, 1996; Bilang-Bleuel et al., 2002; Guzowski, 2002; He et al., 2002; Herry and Mons, 2004; Katche et al., 2010; Stafford et al., 2012; Reul, 2014; Saunderson et al., 2016). On the other hand, there is strong evidence for a major involvement of D2R in DA-dependent induction of striatal c-fos expression (Badiani et al., 1999; Bertran-Gonzalez et al., 2008; Kharkwal et al., 2016), thus these finding indirectly support a role for reduced D2R availability and reduced FSt-induced c-fos-expression in the left DLS of FR mice.

In a final set of experiments we directly tested the hypothesis that reduced D2R availability in the left DLS impair consolidation of passive coping acquired in FSt. To this aim we infused either a D2/D3 or of a D1 receptors antagonist in the left DLS

immediately after the first FSt experience because post-training manipulations influence the consolidation of learning as long-term memory without affecting retrieval (McGaugh, 2000). The results obtained demonstrated that selective blockade of D2/D3R in the left DLS prevents 24 h retention of the immobility response acquired in the first FSt experience by FF mice. Indeed unilateral infusion of sulpiride, but not of SCH23390, in the left DLS dose-dependently reduced immobility expressed on the retention test performed 24 h later.

It should be pointed out that although sulpiride is a mixed D2/D3 receptors antagonist, there are very few D3R in the DLS (Murray et al., 1994; Erritzoe et al., 2014). Moreover, stimulation of D3R has been shown to interfere with all types of learning whereas stimulation of D2R facilitates consolidation of lasting memory (Sigala et al., 1997; Manago et al., 2009; Nakajima et al., 2013; Vicente et al., 2016). Therefore, the findings of this set of experiments offer support to a causal relationship between reduced availability of D2R in the left DLS of FR mice and impaired consolidation of coping acquired in FSt.

CONCLUSION

The results of the present study support the conclusion that reduced dorsal striatal D2R availability fostered by a proximal adverse experience in adult mice can disrupt consolidation of newly acquired coping strategies leading to expression of dysfunctional coping on subsequent encounters with the stressor.

This conclusion has high translational value. Indeed, unseasonal reduction of food availability represents an unpredictable stressor in natural settings (Wingfield and Kitaysky, 2002), thus it models an ecologically meaningful stressor in laboratory settings. Moreover, altered learning mechanisms have been involved in the development of different behavioral disturbances (Robbins and Everitt, 2002; Goodman et al., 2014; Gillan et al., 2016). In addition, low striatal D2R availability has been reported in behavioral disturbances not associated with experience of addictive drugs (Wang et al., 2001; Denys et al., 2004; Schneier et al., 2007; Perani et al., 2008; Tomasi and Volkow, 2013; Broft et al., 2015). Finally, there is strong evidence that the brain circuit supporting consolidation of passive coping in FSt is also involved in response extinction (Campus et al., 2015, 2016; Goodman et al., 2016), suggesting a general role of reduced dorsal striatal D2R in liability to relapse into maladaptive behavior.

AUTHOR CONTRIBUTIONS

PC and SiC designed the research and analyzed and interpreted the data; PC and CO performed behavioral experiments; PC performed c-fos experiments as well as pharmacological experiments, MF and SoC collected, analyzed and interpreted data on striatal levels of D2R mRNA; SP-A, CO, and MF revised the work critically for important intellectual content. SiC wrote the manuscript.

FUNDING

This study was funded by Sapienza Università di Roma (Ateneo 2015; 2016).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 22 September 2017
doi: 10.3389/fphar.2017.00663

Impaired Spatial Memory and Enhanced Habit Memory in a Rat Model of Post-traumatic Stress Disorder

*Jarid Goodman and Christa K. McIntyre**

School of Behavioral and Brain Sciences, University of Texas at Dallas, Richardson, TX, United States

High levels of emotional arousal can impair spatial memory mediated by the hippocampus, and enhance stimulus-response (S-R) habit memory mediated by the dorsolateral striatum (DLS). The present study was conducted to determine whether these memory systems may be similarly affected in an animal model of post-traumatic stress disorder (PTSD). Sprague-Dawley rats were subjected to a "single-prolonged stress" (SPS) procedure and 1 week later received training in one of two distinct versions of the plus-maze: a hippocampus-dependent place learning task or a DLS-dependent response learning task. Results indicated that, relative to non-stressed control rats, SPS rats displayed slower acquisition in the place learning task and faster acquisition in the response learning task. In addition, extinction of place learning and response learning was impaired in rats exposed to SPS, relative to non-stressed controls. The influence of SPS on hippocampal spatial memory and DLS habit memory observed in the present study may be relevant to understanding some common features of PTSD, including hippocampal memory deficits, habit-like avoidance responses to trauma-related stimuli, and greater likelihood of developing drug addiction and alcoholism.

Keywords: single prolonged stress, anxiety, extinction, multiple memory systems, hippocampus and memory, dorsolateral striatum, PTSD

OPEN ACCESS

Edited by:
Antonella Gasbarri,
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Reviewed by:
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Brandi Ormerod,
University of Florida, United States

***Correspondence:**
Christa K. McIntyre
christa.mcintyre@utdallas.edu

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *21 July 2017*
Accepted: *06 September 2017*
Published: *22 September 2017*

Citation:
Goodman J and McIntyre CK (2017) Impaired Spatial Memory and Enhanced Habit Memory in a Rat Model of Post-traumatic Stress Disorder. Front. Pharmacol. 8:663. doi: 10.3389/fphar.2017.00663

INTRODUCTION

Emotional arousal has a dramatic impact on the function of memory systems in the mammalian brain. The memory systems that have been examined in this regard include a cognitive spatial memory system dependent on hippocampal function and a stimulus-response (S-R)/habit system dependent on function of the dorsolateral striatum (DLS). In studies employing animals (e.g., rats and mice) and human subjects, very high levels of emotional arousal have been associated with impairments in hippocampus-dependent spatial memory and enhancements in DLS-dependent habit memory (for reviews, see Packard and Goodman, 2012, 2013; Schwabe, 2013; Goodman et al., 2017a). In addition, in learning situations that can be solved with either spatial or habit memory, stress biases animals and humans toward the use of a DLS-dependent habit strategy (Packard and Wingard, 2004; Schwabe et al., 2007, 2008; Elliott and Packard, 2008; Dias-Ferreira et al., 2009).

Several investigators have suggested that findings pertaining to the emotional modulation of memory are relevant to understanding a variety of human psychopathologies, in particular those involving high levels of stress and anxiety (e.g., McGaugh, 2004; Schwabe et al., 2011; Goodman et al., 2014; Goodman and Packard, 2016a,b). For instance, subjects with post-traumatic stress disorder (PTSD) demonstrate impairments in spatial memory function (Tempesta et al., 2012; Smith et al., 2015; Miller et al., 2017), as well as heightened avoidance responses to trauma-related stimuli (e.g., running away from a loud noise), which may be viewed as an exemplar of enhanced stimulus-response (S-R)/habit memory (for review, see Goodman et al., 2012). Some researchers have proposed that these PTSD symptoms may be partially attributed to the effects of emotional arousal, i.e., stress stemming from the traumatic event, on the hippocampus and dorsolateral striatum (Packard, 2009; Schwabe et al., 2010b; Goodman et al., 2012).

Although the emotional modulation of memory observed in laboratory settings resembles the development of some PTSD symptoms, efforts to associate experimental findings with PTSD are limited by the stimuli and procedures used for eliciting emotional arousal. Experiments specifically examining the influence of stress/anxiety on hippocampus- and DLS-dependent memory have employed a variety of acute and chronic stressors, including predator odor (Leong and Packard, 2014), restraint stress (e.g., Kim et al., 2001; Sadowski et al., 2009; Taylor et al., 2014), fear-conditioned stimuli (e.g., Leong et al., 2015; Goode et al., 2016), and anxiogenic drugs (e.g., Packard and Wingard, 2004; Wingard and Packard, 2008; Schwabe et al., 2010a; Leong et al., 2012; Goodman et al., 2015). Although these stimuli engender robust increases in emotional arousal, the effects are presumably less severe than a traumatic event that produces PTSD symptoms. This limitation curbs the extent to which such laboratory models may be employed for understanding the development of PTSD and for designing novel treatments for the disorder.

In contrast to the stressors employed in previous studies examining the emotional modulation of memory systems, the single-prolonged stress (SPS) protocol produces a neurobehavioral profile that shares many features with PTSD (for review, see Yamamoto et al., 2009). Rats exposed to SPS display multiple behaviors that resemble PTSD symptoms, including sleep disturbances, exaggerated startle response, and impaired extinction of conditioned fear, among others (Liberzon et al., 1997; Khan and Liberzon, 2004; Imanaka et al., 2006; Yamamoto et al., 2009; Nedelcovych et al., 2015). In the present study, we examined whether SPS influences hippocampus-dependent spatial memory or DLS-dependent habit memory in a manner similar to acute and chronic stressors. Rats were initially subjected to SPS and then 1 week later received training in one of two distinct plus-maze tasks, i.e., a place learning task that invokes hippocampus-dependent spatial memory or a response learning task involving DLS-dependent habit memory. Following initial acquisition, rats also received extinction training to determine whether the impairing effect of SPS on fear extinction reported in previous studies (Liberzon et al., 1997; Yamamoto et al., 2008; Knox et al., 2012) may also be observed for spatial and habit memory.

MATERIALS AND METHODS

Subjects

The present study employed 24 experimentally naïve male Sprague-Dawley rats weighing 350–450 g (Taconic, Hudson, NY, United States). Subjects were housed individually in a temperature-controlled vivarium with a 12 h light-dark cycle (lights on at 7:00 AM). Rats were food-restricted and reduced to 85% of their ad lib weight before the start of maze training. Rats were maintained at this weight throughout training, and water remained freely available in their home cages throughout the study. Animal use in this study was carried out in accordance with the ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Dallas. The protocol was approved by IACUC.

Single-Prolonged Stress (SPS) Procedure

The procedure for SPS was selected based on previous studies examining the effects of SPS on PTSD-like symptoms in rats (e.g., Liberzon et al., 1997; Kohda et al., 2007; Yamamoto et al., 2008; Vanderheyden et al., 2015; for review see, Yamamoto et al., 2009). Rats were initially restrained for 2 h in a plastic cone. Immediately thereafter, they were placed into a circular pool of water (22 inch diameter, 25°C) for 20 min of forced swim. Rats were subsequently allotted a 15-min recuperation period before being exposed individually to diethyl ether vapor (Sigma) until they were anesthetized and unresponsive. Finally, rats were returned to their home cages and remained there for 7 days before the start of maze training.

Maze Apparatus

The present study employed a plus-maze apparatus to examine hippocampus-dependent spatial memory and DLS-dependent habit memory. The apparatus consisted of four arms (23 cm in length, 5.75 cm in width, and 9.6 cm in height) arranged in a cross (+) orientation. A movable piece of Plexiglas was also used to block entry to the arm opposite to the start arm for each trial, creating a T-maze configuration that could be modified between trials. The maze was positioned in the center of a distinct room (separate from the room used for SPS) and was surrounded by various extra-maze visual cues.

Training Procedures

The present training procedures were selected based on extensive previous work examining the influence of stress and anxiety on place and response learning in the plus-maze (e.g., Packard and Wingard, 2004; Wingard and Packard, 2008; Sadowski et al., 2009; Leong et al., 2012, 2015; Goodman et al., 2015). Seven days following the SPS procedure, rats were first habituated to the plus-maze apparatus for 2 days. For each day of habituation, rats were placed into the start arm (i.e., the North arm for the 1st day and the South arm for the 2nd day) and were allotted 5 min to explore the maze. Immediately following each day of

habituation, rats were returned to their home cages with 3 Froot Loops cereal pieces (Kellogs). Rats were monitored to confirm Froot Loop consumption.

Twenty-four hours following habituation, separate groups of rats received maze training in either a place learning task (Experiment 1) or a response learning task (Experiment 2) for 12 days (6 trials/day). For each trial, rats were placed into the start arm (North or South) and had the opportunity to retrieve 1/2 Froot Loop in an opaque food cup at the end of the goal arm (East or West). The start arm sequence was NSSNNS on odd days (Days 1, 3, 5, etc.) and SNNSSN on even days (Days 2, 4, 6, etc.). After reaching the correct food cup and consuming the Froot Loop, or after 120 s had elapsed, the rat was removed from the maze. For the intertrial interval (ITI; 30 s), the rat was placed in a holding cage which was located behind a curtain, preventing the rat from viewing the maze during the ITI. For each trial, a correct response was recorded if the rat made an initial full-body entry into the arm containing the food, and an incorrect response was recorded if the animal entered the arm that did not contain the food. The proportion of correct turning responses over the course of training served as a measure of acquisition.

In experiment 1, rats previously given SPS ($n = 6$) or no stress ($n = 6$) were trained in a place learning task involving hippocampus-dependent spatial memory (Packard, 1999; Schroeder et al., 2002; Chang and Gold, 2003; Colombo et al., 2003; Compton, 2004; Jacobson et al., 2012). For each trial of the place learning task, food reinforcement was located in a consistent spatial location (i.e., in the food cup at the end of the West arm). Thus, rats acquired a spatial cognitive map of the learning environment to guide behavior from various start arms to the correct spatial location.

In experiment 2, a separate group of rats was trained in a response learning task invoking DLS-dependent habit memory (Packard and McGaugh, 1996; Chang and Gold, 2004; Palencia and Ragozzino, 2005; Asem and Holland, 2015; for reviews see, Packard and Goodman, 2017). Half the rats were previously given SPS ($n = 6$), and the other half received no stress procedures (i.e., the control group; $n = 6$). For each trial of the response learning task, food was located in the either the East or West goal arm depending on the rat's starting position. When rats started from the North arm, food reinforcement could be found in the East arm, and when rats started from the South arm, food reinforcement was in the West arm. Thus, regardless of their starting position, rats were reinforced to acquire a consistent left turn response at the maze intersection to quickly obtain reinforcement. Previous evidence indicates that stress/anxiety impairs memory in the place and response learning tasks (Packard and Wingard, 2004; Wingard and Packard, 2008; for recent review, see Goodman et al., 2017a).

Twenty-four hours following the last day of acquisition, rats received extinction training (5 days for Experiment 1, and 4 days for Experiment 2). Extinction training was conducted in a manner identical to initial acquisition in each task, except the maze no longer contained food reinforcement. The proportion of correct turning responses over the course of extinction training served as a measure of extinction.

RESULTS

Experiment 1: Place Learning Task

All statistical procedures were conducted using GraphPad Prism 7. The influence of SPS on acquisition and extinction in the place learning task is depicted in **Figure 1**. A repeated measures ANOVA analyzing the proportion of correct responses between the SPS and control groups over the course of acquisition indicated a main effect of Group [$F(1,10) = 6.353$, $p = 0.030$], main effect of Day [$F(11,110) = 7.667$, $p < 0.000$], and a trend toward a significant Group × Day interaction [$F(11,110) = 1.670$, $p = 0.089$]. Tests of simple main effects using Fisher's LSD indicated that the proportion of correct turning responses differed significantly between the SPS and control groups on Training Days 1–3 ($p < 0.05$), whereas the groups did not differ significantly on Days 4–12 ($p > 0.05$). These findings suggest that early in training rats previously receiving SPS were impaired in acquisition of place learning, relative to rats in the control group; however, later in training, SPS and control rats showed comparable memory performance.

A repeated measures ANOVA analyzing the proportion of correct turning responses during extinction indicated a main effect of Day [$F(4,40) = 20.99$, $p < 0.0001$], but no main effect of Group [$F(1,10) = 2.959$, $p = 0.1162$]. However, there was a trend toward a significant Group × Day interaction [$F(4,40) = 2.372$, $p = 0.0685$]. Tests of simple main effects using Fisher's LSD indicated that the proportion of correct turning responses differed between the SPS and control groups during extinction training on Days 15 and 16 ($p < 0.05$). These findings suggest that animals previously receiving SPS were slightly impaired in extinction of place learning, relative to the control group.

Experiment 2: Response Learning Task

The influence of SPS on acquisition and extinction in the response learning task is depicted in **Figure 2**. A repeated measures ANOVA analyzing the proportion of correct responses between the SPS and control groups over the course of acquisition indicated a main effect of Group [$F(1,10) = 6.627$, $p = 0.0277$], main effect of Day [$F(11,110) = 12.11$, $p < 0.0001$], and no Group × Day interaction [$F(11,110) = 1.226$, $p = 0.2783$]. Tests of simple main effects using Fisher's LSD indicated that the proportion of correct turning responses differed significantly between the SPS and control groups on Training Days 3–5 ($p < 0.05$), whereas groups did not differ significantly from each other on Days 1–2 or 6–12 ($p > 0.05$). These findings indicate that rats previously receiving SPS demonstrated an enhancement in the acquisition of response learning, relative to control rats. This enhancement was mainly observed early in acquisition, whereas the SPS and control rats demonstrated comparable performance later in training.

A repeated measures ANOVA analyzing the proportion of correct turning responses during extinction indicated a main effect of Day [$F(3,30) = 33.32$, $p < 0.0001$], but no main effect of Group [$F(1,10) = 1.570$, $p = 0.2387$]. However, we did observe a significant Group × Day interaction [$F(3,30) = 2.974$,

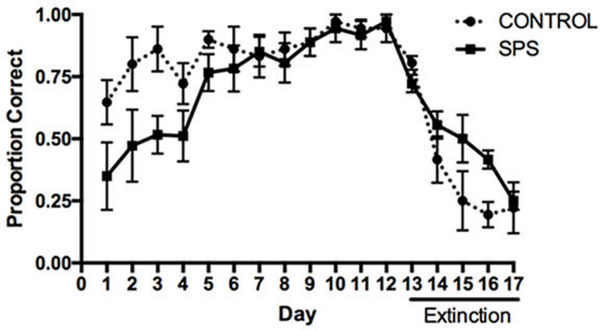

FIGURE 1 | Influence of single-prolonged stress (SPS) on acquisition and extinction of hippocampus-dependent place learning in the plus-maze. SPS rats displayed a lower proportion of correct turning responses during acquisition (Days 1–12), relative to control rats, indicating an impairment of place learning. SPS rats were also slower to reduce the proportion of correct turning responses during extinction training (Days 13–17), indicating an impairment in extinction of place learning.

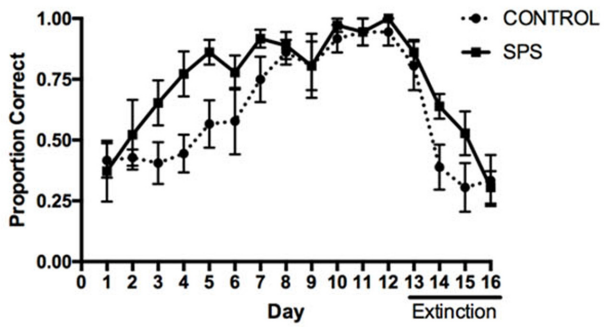

FIGURE 2 | Influence of SPS on acquisition and extinction of dorsolateral striatum (DLS)-dependent response learning in the plus-maze. SPS rats demonstrated a greater proportion of correct turning responses during acquisition of response learning, compared to control rats, indicating an enhancement of response learning. In contrast, SPS rats were slower to reduce the number of correct turning responses during extinction training (Days 13–16), indicating that extinction of response learning was impaired.

$p = 0.0473$]. Tests of simple main effects using Fisher's LSD indicated that the proportion of correct turning responses only differed between the SPS and control groups during extinction training on Day 14 ($p = 0.0439$), whereas there was a trend for a difference on Day 15 ($p = 0.0717$). These results suggest that, relative to the control group, rats previously receiving SPS were impaired in extinction of response learning.

DISCUSSION

The present experiments suggest that multiple memory systems demonstrate different levels of functioning in a rat model of PTSD. Specifically, the SPS protocol, which produces PTSD-like symptoms in rats, may either enhance or impair learning depending on the type of memory being acquired. In Experiment 1, rats exposed to SPS demonstrated impaired acquisition of place learning in the plus-maze, relative to control rats. In contrast, in Experiment 2, rats given SPS showed enhanced acquisition of response learning, compared to controls. Despite differences in initial acquisition of place and response learning, SPS rats showed slower extinction relative to control rats in both the place and response learning tasks, suggesting a general impairment in extinction learning.

Extensive previous evidence indicates that acquisition of place and response learning depend on distinct memory systems. Place learning is mediated by hippocampus-dependent spatial memory, whereas response learning is mediated by DLS-dependent habit memory (for review, see White et al., 2013). Thus, the influence of SPS on performance in these tasks may be attributed to its modulation of hippocampus- and/or DLS-dependent memory systems. Importantly, the place and response learning tasks share similar non-mnemonic (e.g., motivational, motor, sensory, etc.) processes. Therefore, the differential influence of SPS on acquisition in the place and response learning tasks cannot be explained by a potential effect of SPS on non-mnemonic factors.

The present findings are in agreement with previous literature regarding the effects of emotional arousal on multiple memory systems. Acute and chronic behavioral stressors, as well as anxiogenic drugs, typically enhance hippocampus-dependent spatial/cognitive memory, while facilitating DLS-dependent habit memory (for reviews, see Packard and Goodman, 2012, 2013; Schwabe, 2013; Goldfarb and Phelps, 2017; Goodman et al., 2017a). The present results provide evidence that a prolonged stress protocol that produces PTSD-like symptoms in rats may influence memory systems in a similar manner. The present study is also consistent with prior evidence that SPS produces spatial memory impairments in the Morris water maze (e.g., Harvey et al., 2003; Kohda et al., 2007; Wang et al., 2010). It should be noted that other animal models of PTSD have been associated with long-term effects on memory systems (Baratta et al., 2007, 2008; Andero et al., 2011, 2012). Whether these alternative models influence spatial and habit memory in a manner similar to SPS should be examined in future research.

Although we did not examine the mechanisms by which SPS modulates these memory systems, the basolateral complex of the amygdala (BLA) may be involved. The BLA has been implicated in emotional modulation of hippocampus and DLS-dependent memory (Packard and Wingard, 2004; Wingard and Packard, 2008), and evidence indicates that SPS is associated with heightened BLA neural activity (Knox et al., 2016), as well as increased dendritic arborizations of BLA pyramidal neurons (Cui et al., 2008). Increased BLA activity may impair spatial memory via disrupting hippocampal synaptic plasticity (Akirav and Richter-Levin, 1999), i.e., a putative neural substrate of spatial learning (Martin et al., 2000). SPS also leads to downregulation of NMDA receptors in the hippocampus (Harvey et al., 2004), and this mechanism might explain the impairment in spatial memory in the present study, considering that NMDA receptor activation is required for acquisition of place learning in the plus-maze (Mackes and Willner, 2006; Watson and Stanton, 2009; Pol-Bodetto et al., 2011).

It is also possible that SPS influences the function of multiple memory systems via modulating the competitive interaction between hippocampus- and DLS-dependent memory (Poldrack and Packard, 2003). Competitive interactions between memory systems may be observed when disrupting the function of one system facilitates memory acquisition mediated by another system. For instance, several studies indicate that in some learning situations temporary or permanent lesion of the hippocampus leads to faster acquisition of habit memory (Packard et al., 1989; Kim et al., 2001; Schroeder et al., 2002). Moreover, several studies suggest that stress and anxiety may similarly facilitate habit memory via impairing hippocampal memory function (Wingard and Packard, 2008; for reviews, see Packard, 2009; Packard and Goodman, 2012, 2013; Goldfarb and Phelps, 2017). Considering that in the present study SPS impaired acquisition of place learning, and that this SPS protocol has also been associated with spatial memory impairments in other studies (Harvey et al., 2003; Kohda et al., 2007; Wang et al., 2010), disruption of hippocampal memory may constitute part of the mechanism allowing SPS to enhance DLS-dependent response learning in the plus-maze. Future research should investigate the precise neural mechanisms through which SPS modulates memory in the place and response learning tasks, in particular the role of the BLA, hippocampus, and DLS.

The differential influence of emotional arousal on memory systems is purportedly relevant to understanding the etiology of some clinical disorders, in particular those involving stress/anxiety, habit-like behavioral symptoms, and cognitive memory deficits. Disorders discussed in this context include obsessive-compulsive disorder, drug addiction, autism spectrum disorders, Tourette's syndrome, and PTSD, among others (White, 1996; Packard, 2009; Schwabe et al., 2011; Goh and Peterson, 2012; Goodman et al., 2012, 2014; Everitt and Robbins, 2013; Gillan and Robbins, 2014). However, previous studies have employed stressors that neglect important aspects of PTSD pathology. The present study demonstrates that impaired spatial memory and enhanced habit memory may also be observed in a validated rat model of PTSD.

Importantly, the present findings are consistent with some features of PTSD. Indeed, PTSD has been associated with reduced hippocampal volume (Bonne et al., 2001; De Bellis et al., 2001; Fennema-Notestine et al., 2002; Pederson et al., 2004; Golier et al., 2005; Bremner, 2007), as well as impairments in hippocampus-dependent declarative memory (for review, see Bremner, 2006). Likewise, subjects with PTSD show differences in the structure and activity of the dorsal striatum, relative to healthy controls (for review, see Goodman et al., 2012), as well as greater connectivity between the hippocampus and striatum (Rangaprakash et al., 2017). Investigators have suggested that heightened avoidance to trauma-related cues may be regarded as an augmented, maladaptive form of S-R habit memory (Packard, 2009; Goodman et al., 2012). Moreover, subjects with PTSD demonstrate a greater likelihood of developing habit-like comorbidities, such as drug addiction and alcoholism (Jacobsen et al., 2001; McCauley et al., 2012). Whether avoidance symptoms and comorbid drug addiction/alcoholism are related to an enhancement of DLS-dependent habit memory in PTSD remains unknown.

Post-traumatic stress disorder subjects also show impairments in extinction of conditioned fear (Wessa and Flor, 2007; Milad et al., 2008), and this finding has been replicated in rats following exposure to SPS (Yamamoto et al., 2008; Knox et al., 2016). The present findings suggest that extinction of hippocampal spatial memories and DLS habit memories may also be impaired in this rat model of PTSD, even though initial learning of the spatial task was impaired and initial learning of the habit task was enhanced. Downregulation of NMDA receptors in the hippocampus following SPS (Harvey et al., 2004) may explain the impairment in spatial memory extinction, given that this kind of learning is dependent on activation of hippocampal NMDA receptors (Goodman et al., 2016). Extinction of response learning is mediated by the DLS and similarly involves NMDA receptor activity (Goodman et al., 2016, 2017b), however, the influence of SPS on NMDA receptor function and expression in the DLS has not been examined. In addition, whether extinction of spatial and habit memory is impaired in PTSD subjects will also require further investigation.

The persistence of stress and avoidance in PTSD may be due to the enhancement of initial learning or the failure to extinguish learned associations and responses. The present findings indicate that DLS-dependent habit learning is enhanced in a rat model of PTSD, suggesting that conditioned responses may be more difficult to overcome after experiencing a trauma. Results also suggest that extinction of appetitive learning is impaired, independent of whether the initial learning was enhanced or not. These findings are consistent with others demonstrating impairments in the extinction of contextual and auditory fear conditioning (Yamamoto et al., 2008; Knox et al., 2012), and they show that extinction impairments are not specific to fear learning in the rat model of PTSD.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the National Institutes of Health's Office of Laboratory Animal Welfare. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

AUTHOR CONTRIBUTIONS

The research was carried out by JG when he was a *post doc* in CM's lab. The study was designed by both authors. JG did the data analysis and wrote the first draft of the manuscript. CM edited and added to the manuscript.

FUNDING

This work was sponsored by the National Institute of Mental Health MH105014 (CM) and MH104384 (CM).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 04 October 2017
doi: 10.3389/fphar.2017.00701

Reading a Story: Different Degrees of Learning in Different Learning Environments

*Anna Maria Giannini[1], Pierluigi Cordellieri[1] and Laura Piccardi[2,3]**

[1] Department of Psychology, Sapienza University of Rome, Rome, Italy, [2] Department of Life, Health and Environmental Sciences, University of L'Aquila, L'Aquila, Italy, [3] Neuropsychology Unit, IRCCS Santa Lucia Foundation, Rome, Italy

The learning environment in which material is acquired may produce differences in delayed recall and in the elements that individuals focus on. These differences may appear even during development. In the present study, we compared three different learning environments in 450 normally developing 7-year-old children subdivided into three groups according to the type of learning environment. Specifically, children were asked to learn the same material shown in three different learning environments: reading illustrated books (TB); interacting with the same text displayed on a PC monitor and enriched with interactive activities (PC-IA); reading the same text on a PC monitor but not enriched with interactive narratives (PC-NoIA). Our results demonstrated that TB and PC-NoIA elicited better verbal memory recall. In contrast, PC-IA and PC-NoIA produced higher scores for visuo-spatial memory, enhancing memory for spatial relations, positions and colors with respect to TB. Interestingly, only TB seemed to produce a deeper comprehension of the story's moral. Our results indicated that PC-IA offered a different type of learning that favored visual details. In this sense, interactive activities demonstrate certain limitations, probably due to information overabundance, emotional mobilization, emphasis on images and effort exerted in interactive activities. Thus, interactive activities, although entertaining, act as disruptive elements which interfere with verbal memory and deep moral comprehension.

Keywords: multimedia, learning scenarios, verbal memory, visual memory, moral comprehension

OPEN ACCESS

Edited by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico

Reviewed by:
Tim Niclas Höffler, IPN – Leibniz-Institut für die Pädagogik der Naturwissenschaften und Mathematik, Germany
Jing Zhao, Capital Normal University, China

***Correspondence:**
Laura Piccardi
laura.piccardi@cc.univaq.it

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *26 June 2017*
Accepted: *20 September 2017*
Published: *04 October 2017*

Citation:
Giannini AM, Cordellieri P and Piccardi L (2017) Reading a Story: Different Degrees of Learning in Different Learning Environments. Front. Pharmacol. 8:701. doi: 10.3389/fphar.2017.00701

INTRODUCTION

Narrative language is a complex form of discourse that conveys information related to action, narrated events, and the internal states of the characters interacting in the story. Generally, narrative comprehension is an important step in human development and experience. Children's ability to comprehend fictional narratives is related to three key aspects of the story: causal relationships in stories, goals and internal states of the characters in the stories, and integration of the different parts of the stories (Bruner, 1986; Bamberg, 1987; Hudson and Shapiro, 1991; Trabasso and Stein, 1997; van den Broek, 1997; Hickman, 2004; Rollo, 2007). The listener (or reader) has the expectation of logical coherence (cause and effect) between events (Graesser et al., 1980; Barthes, 1981). In general, narrative comprehension involves many perceptual and cognitive sub-processes, including perceiving individual words, parsing sentences, and understanding the relationships between characters (Wehbe et al., 2014). When a child reports events and facts from a story, he/she uses specific words that refer to internal states such as perceptions, emotions, and

desires; very often, the child puts him/herself in the shoes of the main character (Baumgartner and Devescovi, 2001; Rollo, 2007; D'Amico et al., 2008). Children learn about characters, events and values through the spoken communication, miming and gestures of a narrator, or by reading texts directly in the simplest and most linear forms (texts are defined as *monomedia* when they use only writing or only illustrations, or as *bimedia* when writing and illustrations are combined). Trends in educational methods and in entertainment mean that children's learning and memorization are limited to the abovementioned traditional methods. Currently, the development of "multimedia" techniques with the presentation of texts (and *hypertexts*) on computer screens (Jonassen, 1988; Landow, 1992; Furió et al., 2013; Butcher, 2014; Clark and Feldon, 2014) has extended educational methods. Critical approaches focus on the motivational aspects of multimedia procedures, which involve the implicit or explicit invitation to browse, explore and extend information, as well as to master the activities proposed by a computer. The satisfaction gained in this way makes the activity fun and pleasant according to some researchers (e.g., Furió et al., 2013), though it can also end up diverting children's attention and affecting their learning and/or remembering of the proposed core content, particularly verbal information (for a critical review on motivational components see De Beni and Moè, 2000; Giannini, 2002; Mayer, 2014a). In particular, Kashihara et al. (2000) found that multimedia learners who are confronted with motivational elements may be distracted from information processing, with consequences for cognitive learning.

In studies which compare learning from traditional books and learning from computer screens it has been shown that text and illustrations may be more effective than narrated animations (Mayer et al., 2005). It is known, for instance, that externalizing a story improves children's memory for that story (Glenberg et al., 2004) as well as internalizing a text through emotional expression and gesture (Noice and Noice, 2006). These results are also supported by neuroimaging studies that demonstrate that among the variety of brain regions that encode information about story characters, characters' physical movements are represented in brain regions (i.e., the posterior temporal cortex/angular gyrus) that are implicated in the perception of biological motion (Grossman and Blake, 2001; Wehbe et al., 2014) and related to mental motor imagery. Other studies have investigated how the types of illustrations used influence how much children generalize after having read an illustrated book (Ganea et al., 2009; Tare et al., 2010). Children seem to learn more from illustrated books with realistic photographs or color drawings than simple line drawings (Simcock and DeLoache, 2006, 2008). However, the efficacy of illustrations and animations as tools for improving learning remains a controversial area; for example, conflicting results have been obtained by Lowe, who found learning facilitation with animations (Lowe and Schnotz, 2014; Ploetzner and Lowe, 2014; Lowe, 2015). Narrative memory presented in written or verbal form is enhanced by pictures (Levin and Lesgold, 1978; Brookshire et al., 2002; Carney and Levin, 2002) because exposure to information both verbally and pictorially provides redundant retrieval routes (Paivio, 1970, 1986). Pictures may also enhance attention to and comprehension or organization of material, or they may provide cues about important information in the text to keep activated. All of these factors may promote the formation of stronger, more elaborate and more organized memory trace (Gernsbacher, 1990; Levin and Mayer, 1993). Indeed, according to the seminal "dual coding" theory of Paivio (1991), there are two major systems engaged by the presentation of information: one related to verbal and linguistic stimuli and the other related to visual information and mental images. According some authors, multimedia presentation produces a beneficial effect on learning thanks to the "dual coding" hypothesized by Paivio. However, although the advantage of learning through multimedia is now accepted, there is still debate as to whether multimedia presentation is the optimal approach for giving instructions and learning content (Kalyuga et al., 1999; Higgins et al., 2009; Eitel et al., 2013; Arndt et al., 2015). Indeed, in some cases, multiple verbal and non-verbal presentations may add to the "cognitive load" of the user (Chandler and Sweller, 1991; Paas and Sweller, 2014). This especially applies to information presented redundantly. Sensory channel encoding has limited resources, and it is therefore necessary to avoid situations involving excessive cognitive load. According to cognitive load theory (Sweller, 1988), to facilitate changes in long term memory related to schema acquisition, it is necessary to reduce the cognitive load of learners to a minimum. One way to reduce cognitive load is by becoming increasingly familiar with the material. Familiarity alters the cognitive characteristics associated with the material. This promotes schema acquisition, making it easier to handle the material in working memory. Indeed, cognitive load results from several elements being held and manipulated simultaneously in working memory (Sweller, 1994). Unfortunately, working memory is a finite resource that can be overloaded; to overcome this limit, it is necessary to organize learned information into schemas. This organization, as mentioned above, allows more efficient learning. Learning is undoubtedly more lasting and durable when learners are cognitively engaged in the learning process (Bransford et al., 2000; Chinn, 2011). Accordingly, learning environments are most effective when they elicit effortful cognitive processing by guiding learners in actively constructing meaningful relationships rather than encouraging passive recording and storage of information (Craik and Tulving, 1975; Wittrock, 1992). This is the concept of "active development," that recognizes the "importance of active participation of the student, who must necessarily act on the material presented through operations such as selection of the most meaningful information, the organization in an appropriate mental representation, and integration with the knowledge previously acquired, enabling the consolidation in long-term memory" (Wittrock, 1992). Some authors (Mayer and Moreno, 1998; Moreno and Mayer, 2000; Mayer, 2001, 2005, 2011, 2014b) have proposed a theoretical model based on this concept. The methods for achieving "active development" have been described by Mayer and are mainly related to respecting just a few rules. Among these rules is the spatial and temporal proximity of different signs: we learn better when corresponding words and pictures are presented physically close to each other and at the same time, or at least in sequence. This principle

stems from dual coding theory (Paivio, 1971, 1991; Clark and Paivio, 1991; Mayer and Anderson, 1991), which suggests the importance of avoiding redundant forms that encumber attentive efforts and evaluating the message, especially by considering a criterion of consistency of information. Multimedia formats often do not take into account these considerations, resulting in reduced quality in the forms of learning, particularly in terms of narrative thought. Stories are a flexible language to interpret and talk about reality, but they still require structural continuity to be properly understood.

In the present study, we aimed to investigating narrative comprehension acquired through different learning environments (traditional illustrated books: TB; stories displayed on a PC screen enriched with interactive activities: PC-IA; or stories displayed on a PC screen but not enriched with interactive activities: PC-NoIA). In particular, we were interested in better understanding which story content (verbal content, visual details and moral) is advantaged when children read and incidentally learn a story through different learning environments that represent different modalities of presentation (i.e., written in a book or displayed on a PC screen with or without interactive elements). To achieve this purpose, we asked to a large sample of 7-year-old normally developing children to read three different stories acquired in these three different learning environments (TB vs. PC-IA vs. PC-NoIA). In this way, we explored incidental learning by asking children to interact with the story for a fixed time-limit and without asking them to explicitly learn the tale. We expected to observe differential effects on memory according to learning environment: we hypothesized deeper comprehension of the moral meaning as well as increased verbal memory in TB than in PC-IA and increased attention toward visuo-spatial details in PC-IA and PC-NoIA relative to TB. We also hypothesized that adopting two different PC learning environments would allow us to assess the effects of both interactive activities and reading through a PC screen, which requires different eye movement patterns in exploring the text than with traditional books. Furthermore, we reasoned that PC-NoIA may represent a middle ground between traditional learning methods and electronic devices. In such a way, we can investigate whether electronic devices *per se* induce children to pay attention to different features (verbal and/or non-verbal contents) even when interactive activities are not provided.

MATERIALS AND METHODS

Participants

A large sample of 450 seven-year-old children, with both genders equally represented (233 girls, corresponding to 52.5%, and 211 boys, 47.5%) and without reported school difficulties, took part in the study. Knowledge of the three stories used as experimental material (i.e., Aladdin's Lamp; The Three Little Pigs; and Adopting a Star) was an exclusion criterion, while one inclusion criterion was that all pupils had to be able to use a personal computer. Any participant who failed to meet the above-mentioned criteria was excluded from the experiment but was involved in a secondary task consisting of a pleasant reading which was not relevant to the purposes of the study. All participants attended primary schools or the Educational and Sport Centres of the Municipality of Florence (Italy). Each child was examined individually in an appropriate room of his/her school or center. Foreign children and those with learning difficulties and other neurodevelopmental diseases (as reported by their teachers or families) were not included in the study. None had primary visual or hearing impairments or had been diagnosed with a neurological condition.

The examiner subdivided participants in three groups consisting of three different learning environments: (i) individual traditional book reading (TB); (ii) individual reading of the story, displayed on the computer screen and interspersed with interactive activities (PC-IA); and (iii) individual reading of the story, shown on the computer screen but not interspersed with interactive activities (PC-NoIA). The precise sample for the TB condition was 78 girls and 70 boys, with 2 children's gender not indicated; for PC-IA, there were 77 girls and 72 boys, with 1 child's gender not indicated; and for PC-NoIA, there were 78 girls and 69 boys, with 3 children's gender not indicated.

The three possible stories (Aladdin's Lamp; The Three Little Pigs; and Adopting a Star) were equally distributed across the three learning environments. Furthermore, the difficulty and comprehensibility were balanced across the three stories.

The study was approved by the local ethical committee of the Department of Psychology, Sapienza University of Rome, in accordance with the Declaration of Helsinki. A signed consent form was obtained from parents and an assent from each child. Specifically, the consent obtained from the parents of all research participants was both informed and written.

Materials

We started by looking for editions of children's stories in order to present identical texts and pictures in the three different learning environments (TB, PC-IA, and PC-NoIA). **Figure 1** reports an example taken from the book "Adopting a Star." For each learning environment, corresponding to a different modality of story presentation, children read only one story. In the PC-NoIA condition the modality of story presentation was the same as in TB, and children were asked to move through the story using the mouse. The child chose to go forward or backward by clicking on two arrows, as if leafing through a book. In contrast, in the PC-IA condition various options were available, including listening to narration corresponding to the written text, with different voices for each character, as well as hearing animal noises and answering written questions by ticking boxes with the mouse.

We selected adaptations and re-editions of the following stories that had been published in Italy: (1) *La Lampada di Aladino* ("Aladdin's Lamp") published by Kyberkid, Città di Castello (Maestripieri, 2001); (2) *I Tre Porcellini* ("The Three Little Pigs"), published by Giunti, Florence (Escofet et al., 2000); and (3) *Adottare una Stella* ("Adopting a Star"), published by Edizioni S. Paolo, Cinisello Balsamo, Milan (Mostacchi, 1993).

The text in each story was between 650 and 1350 words, interspersed with color illustrations (ranging from 15 to 26 pictures). The illustrations were carefully checked to assess their relevance to the text, and we also ensured that the spatial

FIGURE 1 | An example from the book "Adopting a Star" of the different modalities. **(A)** Presentation through traditional book (TB); **(B)** presentation through PC-NoIA; **(C)** presentation through PC-IA. The San Paolo publishing house has consented to the use of one picture from the book "Adottare una stella" (1993) by Massimo Mostacchi and illustrated by Monica Miceli, San Paolo Edizioni. The figure is being reproduced with the permission from the copyright holder.

arrangement of text and illustrations in the three versions used was more or less equivalent in terms of surface allotment (see **Figure 1** for an example).

The three stories did not differ in terms of verbal and non-verbal memory details reported independently from the learning environment (verbal details: $F_{2,441} = 1.461$, $p = 0.233$; $\eta_p^2 = 0.006$; observed power = 0.28; non-verbal details: $F_{2,441} = 2.315$, $p = 0.100$; $\eta_p^2 = 0.008$; observed power = 0.40). We also examined gender differences in emotional involvement, liking and interest arousal for each story (see **Table 1** for details), but no gender differences emerged.

For this reason, we merged the three stories in subsequent analyses.

Various interactive options were available, as well as the opportunity for motor activities by actually using the computer itself. Interactive activities included musical accompaniments, voices narrating the written text, different voices for each character in the story, animal noises, the movements of leading and secondary characters, animations of natural events (such as rain and storms, with voices naming them), ideas for games such as puzzles, mazes, revealing masked items, matching spoken and written words with pictures, riddles, constructions, and painting activities.

None of these activities were included in the PC-NoIA condition, which displayed only the written text and illustrations that appeared in the book format.

Procedure

Interaction with each story in each learning environment was limited to approximately 20 min. The examiner did not require children to learn but rather to perform a silent reading of the stories. We preferred to investigate incidental learning derived from the three different learning environments, since this type of learning is more similar to everyday situations in which children of that age peruse written texts, enhancing the ecological validity of the study.

After reading the story, each child was individually required to complete a written test. This testing was unannounced, included 14 written questions and lasted approximately 25 min; the written answers were supplied immediately after children finished the story. Double-blind conditions were maintained throughout the experiment.

The degree of learning each child had achieved was assessed by collecting the written answers to 10 cued recall questions printed on a card. The first five questions concerned the child's memory for important details of the story text, and the following five questions focused on the pictures. The complete set of questions for each of the three stories, subdivided into two categories (primarily verbal memory and primarily non-verbal memory) is given in the Appendix. The order of the questions was constant in each written test.

The questions were worded so that for children of this age, priority was given to visual non-verbal images rich in

TABLE 1 | Gender effect for emotional involvement, liking and interest arousal for each story.

	Females		Males				
1st story	***M***	***SD***	***M***	***SD***	***F***	***P***	η_p^2
Emotional							
Involvement	8.29	2.63	7.59	3.19	2.819	0.141	0.015
Liking	8.26	2.80	7.81	2.84	0.907	0.343	0.006
Interest arousal	8.09	3.03	7.44	3.35	1.517	0.220	0.010
2nd story							
Emotional							
Involvement	8.62	2.25	8.67	2.08	0.015	0.903	0.000
Liking	8.45	2.33	8.31	2.29	0.154	0.695	0.001
Interest arousal	8.66	2.44	8.67	2.49	0.000	0.991	0.000
3rd story							
Emotional							
Involvement	7.94	2.25	7.94	2.21	0.000	0.987	0.000
Liking	7.94	2.18	7.96	2.32	0.003	0.956	0.000
Interest arousal	8.23	2.18	7.91	2.36	0.719	0.398	0.005

M (means), SD (standard deviations) and analysis of variance (F, P, and η_p^2) are reported.

physiognomic properties (Werner, 1940); the written text was basically a support at this point. Indeed, the images further encouraged children to read the written text, thus forming an information flow through the integration of illustrations and words (Schnotz, 2014).

Levels of positive emotional involvement, appreciation and interest arousal were also assessed through three specific questions, each accompanied by a visually perceived evaluation scale ranging from 1 (minimum) to 10 (maximum) points, as shown in the Appendix. Obviously, any "No" answer yielded 0 points (although this outcome never occurred).

To assess whether and to what extent each child had been able to grasp the so-called "moral" of the story (its overall meaning and the ethical teachings each story conveyed) we added an open question: "Have you learned anything from the story?"

This last question also had to be answered in writing and was presented near the end of the 25-min individual written test session. The data afforded by answers to this question allowed us to study the frequency distribution of the answers according to two categories: (a) successful processing of a relevant moral; and (b) unsuccessful processing of a moral, perhaps with intrusion of or emphasis on irrelevant and/or heterogeneous contents.

It should be stressed that in each story the moral was relatively clearly stated. This simplified the scoring, which was based on the agreement of two out of three expert judges that evaluated the pertinence of each answer and the presence of errors, omissions, or intrusions.

RESULTS

Figures 2, 3, and **Tables 2–5** show statistics derived from the collected data. Recall performance was measured according to the number of correct and relevant memories for the first 10 questions (five relating to verbal memories and five relating to non-verbal memories), which were the same for all three stories in the three learning environments (**Figure 2**). An ANOVA showed that the three learning environments produced significant differences in verbal memory recall ($F_{2,441} = 265.37$; $p < 0.001$; $\eta_p^2 = 0.54$; observed power = 0.99). A Duncan *post hoc* test showed that TB produced higher performance ($p < 0.05$) for verbal details than did PC-IA. Verbal details reported for the TB and PC-NoIA conditions did not differ from each other. The TB and PC-NoIA conditions significantly differed from the PC-IA condition ($p < 0.05$) (**Table 2**).

An ANOVA also showed a significant difference between the three learning environments for non-verbal memories ($F_{2,441} = 37.29$; $p < 0.001$; $\eta_p^2 = 0.12$; observed power = 0.91). *Post hoc* Duncan tests showed that recall performance was significantly higher in PC-IA ($p < 0.05$) relative to TB and PC-NoIA. Non-verbal memory recall was also significantly better also for the PC-NoIA condition ($p < 0.05$) relative to TB (**Figure 3**), but in the PC-NoIA condition non-verbal memory recall was significantly worse than that in the PC-IA condition ($p < 0.05$) (see **Table 2**).

Average scores for positive emotional involvement, liking, and interest arousal were calculated on the basis of answers supplied to the three evaluative questions, which focused on affective aspects of children's experiences of the stories. Three separate ANOVAs were performed on positive emotional involvement, liking and interest arousal according to learning environment. Positive emotional involvement was significantly different for the three groups ($F_{2,441} = 7.50$; $p < 0.01$; $\eta_p^2 = 0.33$; observed power = 0.95). Duncan tests showed that these scores were significantly higher in the PC-IA group ($p < 0.05$; **Figure 2**). Scores were slightly lower in the PC-NoIA group and dropped significantly in the TB group ($p < 0.05$). Liking also significantly differed for the three learning environments ($F_{2,441} = 10.53$; $p < 0.001$; $\eta_p^2 = 0.42$; observed power = 0.98). Duncan tests showed that liking was significantly lower for the illustrated TB condition ($p < 0.05$). The three learning environments showed significant differences for interest arousal ($F_{2,441} = 5.74$; $p < 0.01$;

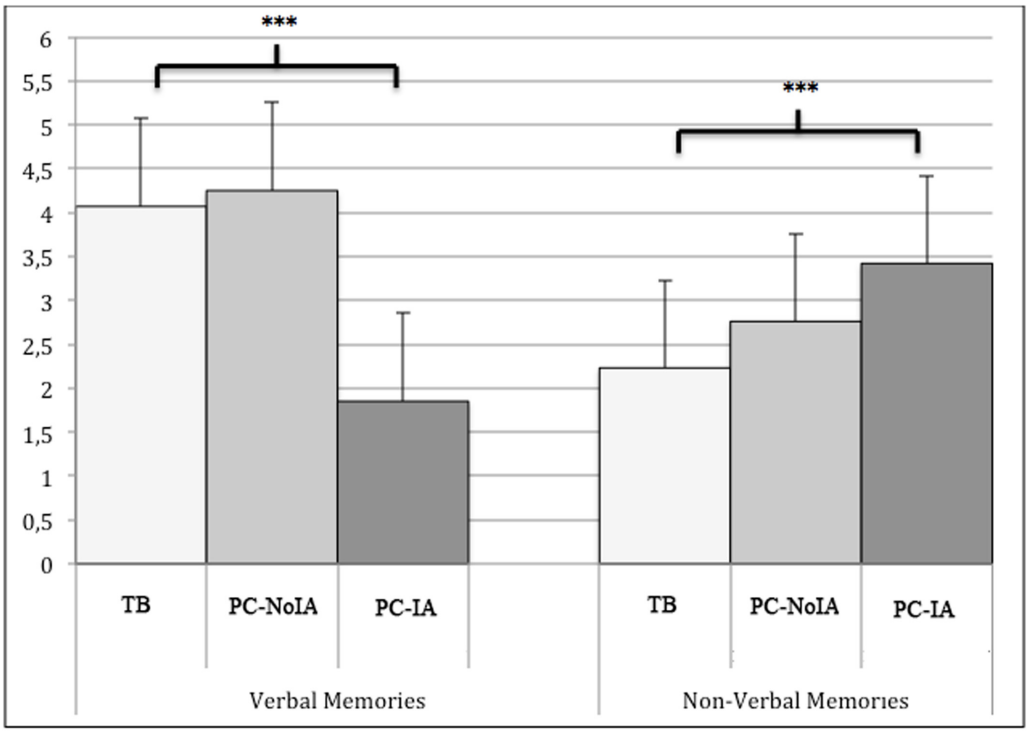

FIGURE 2 | Mean scores for verbal and non-verbal memories in the TB, PC-NoIA, and PC-IA conditions.***$p < 0.001$.

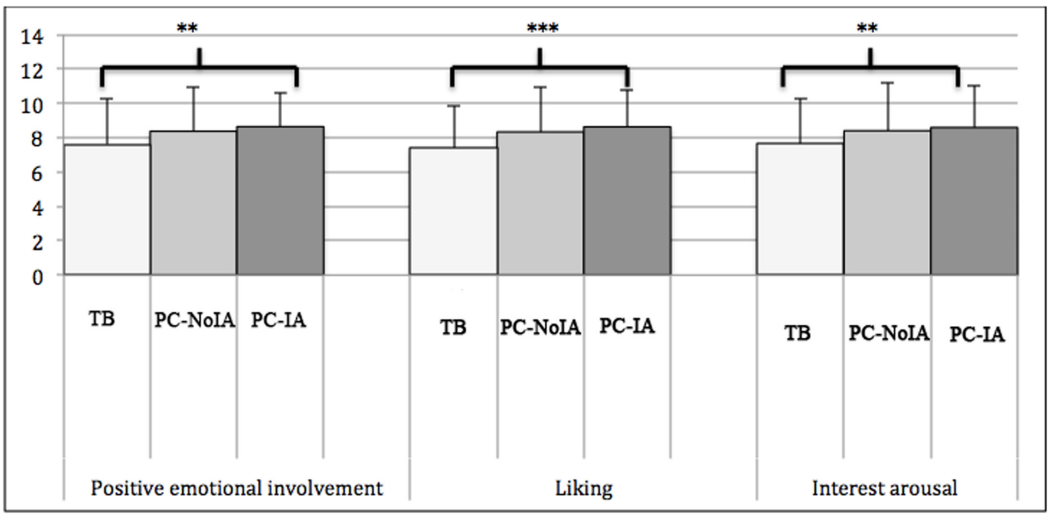

FIGURE 3 | Mean scores for positive emotional involvement and interest arousal in the TB, PC-NoIA, and PC-IA conditions.***$p < 0.001$, **$p < 0.01$.

$\eta_p^2 = 0.24$; observed power = 0.85). Duncan tests indicated a significantly lower score for the TB condition ($p < 0.05$) than the PC-IA and PC-NoIA conditions (**Table 3**).

To ascertain whether multimedia presentation truly made the activity of reading stories fun and pleasant, we performed a Pearson's correlation analysis of verbal and non-verbal details reported in the three different learning environments (TB; PC-IA; and PC-NoIA) with emotions activated by the stories (emotional involvement, liking, and interest arousal). The analysis showed a significant positive correlation between the PC-IA condition and emotional involvement, but only for non-verbal details: specifically, for emotional involvement [$r(147) = 0.164$, $p = 0.047$] and liking [$r(147) = 0.183$, $p = 0.041$] but not for interest arousal. No significant correlations between emotional involvement and verbal details were observed. Interactive modalities had effects on the recollection of non-verbal but

not verbal details. In contrast, in the TB condition emotional involvement and verbal details were positively correlated [$r(145) = 0.193, p = 0.020$]. Interestingly, the PC-NoIA condition demonstrated a positive correlation between verbal details and emotional involvement [$r(152) = 0.169$, $p = 0.037$], arousal interest [$r(152) = 0.172$, $p = 0.034$] and liking [$r(152) = 0.289$, $p = 0.001$]. Non-verbal details were also correlated with emotional involvement [$r(152) = 0.199$, $p = 0.014$], interest arousal [$r(152) = 0.171$, $p = 0.035$] and liking [$r(152) = 0.289$, $p = 0.001$]. Therefore, while the TB condition showed an

TABLE 2 | Overall recall scores for verbal and non-verbal memories.

Groups	*n*	Verbal memories		Duncan's test*	Non-verbal memories		Duncan's test*
		M	*SD*		*M*	*SD*	
TB	150	4.07	0.93	A	2.23	1.23	A
PC-NoIA	150	4.25	1.03	A	2.76	1.13	B
PC-IA	150	1.85	1.08	B	3.42	1.32	C
Analysis of Variance		$F_{2,441} = 265.37$ $p < 0.001$ $\eta_p^2 = 0.54$			$F_{2,441} = 37.29$ $p < 0.001$ $\eta_p^2 = 0.12$		

*M (means) and SD (standard deviations); n (number of participants). *Means with different letters (Duncan's test) are significantly different ($p < 0.05$).*

TABLE 3 | Overall scores for affective involvement.

Group	*n*	Positive emotional involvement		Duncan's test*	Liking		Duncan's test*	Interest arousal		Duncan's test*
		M	*SD*		*M*	*SD*		*M*	*SD*	
TB	150	7.61	2.69	A	7.41	2.45	A	7.63	2.64	A
PC-NoIA	150	8.37	2.53	B	8.33	2.59	B	8.40	2.78	B
PC-IA	150	8.64	1.99	B	8.63	2.16	B	8.59	2.43	B
Analysis of variance		$F_{2,441} = 7.50$ $p < 0.01$ $\eta_p^2 = 0.33$			$F_{2,441} = 10.53$ $p < 0.001$ $\eta_p^2 = 0.42$			$F_{2,441} = 5.74$ $p < 0.01$ $\eta_p^2 = 0.24$		

*M (means) and SD (standard deviations); n (number of participants). *Means with different letters (Duncan's test) are significantly different ($p < 0.05$).*

TABLE 4 | Correlation matrix.

Learning environment	Memory	Emotional involvement	Liking	Interest arousal
TB	Verbal	0.193*	0.116	0.048
	Non-verbal	−0.13	−0.102	−0.04
PC-NoIA	Verbal	0.169*	0.172*	0.289***
	Non-verbal	0.199*	0.102	0.171*
PC-IA	Verbal	0.058	−0.037	−0.064
	Non-verbal	0.164*	0.073	0.183*

*$p < 0.05$, ***$p < 0.001$.*

TABLE 5 | Frequency distribution for responses concerning the morals of the stories.

	First story (Aladdin's Lamp)		Second story (The Three Little Pigs)		Third story (Adopting a Star)	
	Moral	No moral	Moral	No moral	Moral	No moral
TB	37	13	24	26	32	18
PC-NoIA	16	34	31	19	21	29
PC-IA	20	30	8	42	11	39
	$\chi^2 = 19.51$ $p < 0.001$		$\chi^2 = 22.82$ $p < 0.001$		$\chi^2 = 18.04$ $p < 0.001$	

effect only for verbal details and PC-IA for non-verbal details, the intermedia learning environment (PC-NoIA) exhibited significant correlations with both verbal and non-verbal details (see **Table 4** for the correlation matrix). As a consequence, we could hypothesize that emotional involvement, liking and interest arousal are more related to the device (reading on the PC vs. reading a traditional book) than performing additional activities during reading.

Concerning the question of the moral and teaching of each story, there were significant differences in the frequency of answers showing comprehension and retention of the relevant moral (i.e., the core meaning of the story) between groups (i.e., first story: $\chi^2 = 19.51$; $p < 0.001$; second story: $\chi^2 = 22.82$; $p < 0.001$; third story: $\chi^2 = 18.04$; $p < 0.001$). Specifically, the TB group was better at understanding the moral of the story ($p < 0.01$), whereas this frequency was lower in the PC-IA group. The PC-NoIA condition yielded intermediate results (see **Table 5**).

We observed qualitative differences in responses concerning the stories' morals produced in the three learning environments. Here, we report examples of answers provided in the different learning environments. Specifically, children in the PC-IA group produced more irrelevant content, which generally referred directly to computer use, as opposed to story contents: for example, "I learnt to click . . ." (to use a mouse); "The arrows on the keyboard" (the cursors for turning the page); "That you turn over the page using the mouse"; "You can move things, trees, the dog, people, etc."; "I learnt the maze", and "The puzzle..." (or other story-related play activities). In contrast, children's answers when they learnt through TB were more often pertinent, such as the following: "You mustn't trust strangers"; "You must be careful when you suspect something"; "You shouldn't trust strangers even when they promise you something gold"; "You must be righteous, good and never tell lies"; and "It is wrong to steal" (based on reading "Aladdin's Lamp"). In the case of "The Three Little Pigs," children in the TB condition responded: "You should work and be far-sighted in life"; "We must work well"; and "They should have built a brick house all together. . .". For the story "Adopting a Star," responses included: "I learnt that love between people is very important"; "Sometimes we should think of others and not just ourselves"; and "When you find something, you should always ask who it belongs to."

DISCUSSION

Our main aim was to investigate differences in incidental learning produced by different learning environments (TB, PC-NoIA, and PC-IA). We hypothesized that differences in story presentation could induce differences in verbal and non-verbal memories as well as in deep comprehension of the story's moral. With this intent, we analyzed different types of learning effects (verbal and visual memory) as well as moral comprehension, which involves deep comprehension of spiritual and ethical meanings that children will need to make choices and take actions reflective of universally accepted beliefs and values. The novelty of our study was to introduce an intermediate learning environment that did not require participants to perform interactive activities but displayed information on a PC screen, similarly to a book. The introduction of this further learning environment allowed us to better understand the effect of interactive activities on incidental learning. Furthermore, it allowed us to compare traditional teaching with teaching on a novel electronic device without adding any type of activities but only requiring participants to read the story displayed on the PC. Our results showed that children who had dealt with PC screen reading and performing interactive activities reached higher levels of positive emotional involvement, liking, and interest. This result is in line with other studies (e.g., Sun et al., 2008; Ahmadi Gilakjani et al., 2012; Furió et al., 2013, Furió et al., 2015) that found that students reported greater satisfaction and motivation when they learned through new technologies. Additionally, our results showed higher levels of positive emotional involvement, liking, and interest toward the stories presented on the PC with or without interactive activities, relative to TB. At the same time, our sample clearly verbally recalled fewer essential details of the narrative. In contrast, non-verbal memory was enhanced by the information conveyed through illustrations and/or animations, but interactive activities generally did not help in grasping the core meaning of the story, especially its ethical aspects. In other words, the way in which the story has been read contributes to the priority of the elements that are learnt and remembered. This priority was influenced by the appeal of specific elements and by the implicit role of distracting elements. However, in learning environments without interactive activities (PC-NoIA), recall of verbal details was comparable to that acquired through a traditional learning approach (TB), demonstrating that in multimedia presentation, it is very important how content is provided. Interestingly, the PC-NoIA condition, like the PC-IA condition, contributed to improve performance in recalling non-verbal details. However, the PC-NoIA condition exhibited stronger positive correlations than the TB condition with emotional involvement, liking and interest arousal both for verbal and non-verbal memory, suggesting that the new devices *per se* may enhance learning, especially when there are no added activities. Thus, PC presentation promotes attention toward non-verbal details even when they are not required to perform any type of activities. We speculate that this enhancement may be due to exploratory eye movements during reading that differ from those during TB reading. This difference could also be because we use a PC and not a tablet. Indeed, it is also possible that reading an eBook on a tablet is more similar to TB reading both in the way we hold the object by hand and in the ocular scanning performed by individuals. Another possibility may be related to the screen's backlighting, which may increase performance on non-verbal details. Recent studies have demonstrated effects of luminous radiation from visual comfort to psychological and physiological wellbeing (e.g., Vandewalle et al., 2009; Ferlazzo et al., 2014).

Al-Qahtani and Higgins (2013) investigated the effects of e-learning, blended learning (which combines e-learning and traditional teaching), and classroom learning. They found a statistically significant difference between the blended learning method and the other two methods. However, these authors did not find any significant difference between the e-learning and

traditional learning groups. Girard et al. (2013) analyzed studies that compared game-based learning tools with more traditional approaches and found that games had the same learning effect as traditional approaches. In contrast, our study seems to suggest that content requiring a deeper analysis of the text (such as the moral of a story) is reduced when children read the story on a PC, particularly when they are also involved in interactive activities.

A possible interpretation of our results is that interactive activities could act as distracters producing a reduction of attention and a greater focus on a perceptual level, which affects verbal learning. This effect could be attributed to both proactive and retroactive interference effects (i.e., interfering with verbal cognition learned both after and before the reading activity) (e.g., Bower and Mann, 1992; Mayer, 2001, 2005). An interference effect could be due to the need to continually coordinate visual perception and motor skills necessary to read by means of a PC, as well as to the psychological effort (in terms of cognitive load) that the PC interaction may involve. However, the inclusion criterion that children be familiar with PCs, should have reduced such interference, especially considering that all were competent with digital media and did not experience an increase in cognitive load while using a daily tool with which they were high familiar. Indeed, according to cognitive load theory (Sweller, 1988) when a learner is familiar with the material or with the environment, the familiarity effect reduces cognitive load and increases the activation of previously learned schema. However, according to Sweller (1994), learning environments with high interactivity (such as the PC-IA condition) may introduce extraneous cognitive load that can negatively interfere with learning. Another possible explanation is the split-attention effect (e.g., Ayres and Sweller, 2014) between verbal and the visual information as well as due to the entertaining activities children may perform while reading the story reading. These activities, although pertinent, may result in split attention. This explanation is also in line with evidence that reading the text without interactive activities (as in the PC-NoIA learning environment) produced equally detailed verbal recall.

In the present study, although all groups were able to comprehend the pertinent moral, participants that read the story on a PC screen more often gave inappropriate answers, as if to make up for gaps in essential memories. Children's answers thus suggest that they gave priority to memorize irrelevant content, which generally involved computer use, as opposed to aspects of the content. Differences in the ability to identify and describe the moral of the story appeared to be related to differences in recalling verbally coded passages from the story and their interrelations, even if this interpretation does not explain why children exposed to the PC-NoIA condition did not obtain the same level as in the TB condition in comprehending the story's moral, since their performance in remembering verbal details was comparable.

Our data demonstrate instead that using extensive interactive situations can adversely affect recall at a verbal level, thus significantly reducing specific memory performance. On the other hand, the latter increases for image recall on a non-verbal level. In other words, the congruence between memorized material and the recall task seems to matter most, along with possible interference or cooperation between different channels.

Karunanayaka et al. (2007) studied developmental trends in the neural substrates supporting narrative comprehension and found age-related differences in brain activity, which may reflect changes in local neuroplasticity. The authors performed a group-level independent component analysis (ICA) that allowed them to identify the involvement of the following right structures: primary auditory cortex, mid-superior temporal gyrus, the most posterior aspect of the superior temporal gyrus, hippocampus, angular gyrus and the medial aspect of the parietal lobule (precuneus/posterior cingulate). Furthermore, a left-lateralized network was also identified comprising the inferior frontal gyrus (including Broca's area), inferior parietal lobule, and medial temporal lobe. This widespread cerebral network suggests hypotheses concerning functional segregation in Broca's and Wernicke's areas, the crucial role of the right hemisphere in narrative comprehension and increased left hemispheric dominance for language processing with age (Karunanayaka et al., 2007). Neuroimaging data stress the complexity of narrative comprehension, which involves a widespread network of brain areas in both the hemispheres. In line with this evidence, it is possible to hypothesize that differences in learning due to the learning environment in which text is learned may be related to activation of different brain areas. Thus, a traditional book elicits deeper comprehension of the story's meaning, while an interactive book focuses on visual details that are probably elicited by activities that require children to pay attention to a story's visual details. In contrast, written text forces a deeper processing of content, reducing distracting factors and requiring readers not to perform actions in response to the text but only to pay attention to its contents.

Additionally, our results provide practical observations that may be useful for educational techniques. Indeed, these findings contribute to better understanding of how technology interacts with and affects cognitive structures. From a practical point of view, if the aim is to memorize computer procedures and visual images in particular, then it is worth adopting a rich multimedia approach. If, on the other hand, we wish children to learn the core meaning of the story in the best possible way, then TB is still the recommended approach; alternatively, we recommend at least reducing the interactivity of the multimedia approach.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AMG, PC, and LP. Performed the experiments: PC. Analyzed the data: PC. Discuss the results and wrote the paper: LP, PC, and AMG.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphar.2017.00701/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 16 October 2017
doi: 10.3389/fnins.2017.00574

Enhancing Allocentric Spatial Recall in Pre-schoolers through Navigational Training Programme

*Maddalena Boccia[1,2], Michela Rosella[3], Francesca Vecchione[2], Antonio Tanzilli[1], Liana Palermo[4], Simonetta D'Amico[5], Cecilia Guariglia[1,2] and Laura Piccardi[1,3]**

[1] Neuropsychology Unit, IRCCS Fondazione Santa Lucia of Rome, Rome, Italy, [2] Department of Psychology, Sapienza Università di Roma, Rome, Italy, [3] Life, Health and Environmental Science Department, University of L'Aquila, L'Aquila, Italy, [4] Department of Medical and Surgical Sciences, University Magna Graecia, Catanzaro, Italy, [5] Department of Biotechnological and Applied Clinical Science, University of L'Aquila, L'Aquila, Italy

OPEN ACCESS

Edited by:
Alfredo Meneses, Center for Research and Advanced Studies of the National Polytechnic Institute (CINVESTAV), Mexico

Reviewed by:
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Santiago J. Ballaz, Yachay Tech University, Ecuador

***Correspondence:**
Laura Piccardi
laura.piccardi@cc.univaq.it

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *23 June 2017*
Accepted: *02 October 2017*
Published: *16 October 2017*

Citation:
Boccia M, Rosella M, Vecchione F, Tanzilli A, Palermo L, D'Amico S, Guariglia C and Piccardi L (2017) Enhancing Allocentric Spatial Recall in Pre-schoolers through Navigational Training Programme. Front. Neurosci. 11:574. doi: 10.3389/fnins.2017.00574

Unlike for other abilities, children do not receive systematic spatial orientation training at school, even though navigational training during adulthood improves spatial skills. We investigated whether navigational training programme (NTP) improved spatial orientation skills in pre-schoolers. We administered 12-week NTP to seventeen 4- to 5-year-old children (training group, TG). The TG children and 17 age-matched children (control group, CG) who underwent standard didactics were tested twice before (T_0) and after (T_1) the NTP using tasks that tap into landmark, route and survey representations. We determined that the TG participants significantly improved their performances in the most demanding navigational task, which is the task that taps into survey representation. This improvement was significantly higher than that observed in the CG, suggesting that NTP fostered the acquisition of survey representation. Such representation is typically achieved by age seven. This finding suggests that NTP improves performance on higher-level navigational tasks in pre-schoolers.

Keywords: human navigation, normal development, allocentric representation, egocentric representation, spatial orientation training, environmental knowledge, survey knowledge

INTRODUCTION

Representing and transforming spatial information are everyday activities that are crucial to moving to a new town, reading and interpreting maps. Human navigation requires the ability to mentally transform images from two-dimensional to three-dimensional forms, similar to following a map to reach a goal in a new environment. It also requires recognizing a place from a different perspective and finding an alternative route when an initial route is interrupted. Navigational ability and spatial behavior develop gradually and at distinct time points during childhood (Siegel and White, 1975; Lehnung et al., 2003). According to Siegel and White's model 1975, environmental knowledge is acquired in three separate and distinct steps: (i) *landmark knowledge*, with which individuals are able to perceptually discriminate and recognize landmarks but are unable to derive directional information from them (the location of a landmark, its relation with the environment and its relation to other landmarks); (ii) *route knowledge*, with which directional information based on egocentric representation is added to landmark knowledge, allowing individuals to navigate by following directional instructions that link consecutive landmarks (e.g., turn right at the bakery to reach the theater); and (iii) *survey knowledge*, with which

individuals build a mental map of the environment based on an allocentric frame of reference. The way in which environmental knowledge is acquired, as well as its organization continue to be debated. On the one hand, Siegel and White's Model suggests a hierarchical organization in which phases are acquired sequentially (Siegel and White, 1975). On the other hand, Montello (1998) suggests an environmental representation acquired simultaneously. One must distinguish between two different frames of reference in environmental mental representation, namely, egocentric and allocentric representations of the environment. *Egocentric representation* expresses the relation of an environmental object with respect to the self, and this frame of reference is generated from sensory data and may provide a direct basis for action; *allocentric representation* expresses the location of the environmental object with respect to an external frame of reference, which is more difficult to compute but provides a better basis for flexible navigation and long-term storage of complex layouts (Byrne et al., 2007). Egocentric and allocentric representations roughly correspond to the route and survey knowledge described in Siegel and White's Model. In route-based navigation, individuals use egocentric coordinates, whereas in survey-based navigation, they mainly use allocentric coordinates. The nature of spatial representation in the brain indicates the parallel presence of both survey and route knowledge. Individuals could proficiently shift from a route to a survey perspective (Taylor and Tversky, 1992), even if the environment has been learned using the other perspective (Boccia et al., 2016a). Neural activity within the brain network underlying human navigation, such as the parahippocampal place area (PPA) and the retrosplenial cortex (RSC) (Boccia et al., 2014, 2016b), has been found to depend on the familiarization and the type of strategy participants adopt in performing a topographical memory task (Boccia et al., 2016a). These findings suggest that these brain areas underlie the shift between different types of environmental knowledge, namely, route and survey knowledge. This result confirms the idea that environmental objects are processed in parallel in different formats and that a proficient shift from one format to another may occur from the first stage of environmental knowledge acquisition (Montello, 1998).

Regarding the development of navigational abilities during childhood, some competences are developed during the early years, while others require a longer time to become fully functioning. Overall, studies suggest that although some spatial knowledge may be innate, the majority of skills requires time to fully mature (Vasilyeva and Lourenco, 2012; Nys et al., 2015). Some of these skills develop early in life, such as the use of egocentric strategies to find a hidden target, reference memory and visuo-spatial working memory (Piaget and Inhelder, 1948; Acredolo, 1978; Bremner, 1978; Acredolo and Evans, 1980; Foreman et al., 1984, 1990), while others require a longer period to fully develop. For example, the development of navigational working memory is complete at ∼10 years of age (Piccardi et al., 2014b). A similar developmental trajectory holds for the ability to construct a cognitive map of the environment based on distal cues, that is, a stable mental representation of navigational space (O'Keefe and Nadel, 1979). This competence becomes fully developed at approximately only 7 years of age (Overman et al., 1996; Lehnung et al., 1998, 2003). Later, by age 10, relational place strategies, which are necessary for cognitive mapping, develop (Overman et al., 1996; Lehnung et al., 1998). More recent studies suggest that by the age of 4, children use movement information or unique proximal landmarks to solve a viewpoint-independent reorientation task in which spatial recall cannot be performed using a stored view, that is, using a flexible representation of the environmental layout. Instead, a flexible recall from novel viewpoints is available by the ages of 6 to 8 (Nardini et al., 2009). Interestingly, 4-year-old children have some basic features of allocentric coding (Negen et al., 2017). Moreover, the spontaneous use of an allocentric world-centered representation of the environment progressively increases between 5 and 10 years of age (Bullens et al., 2010), even if younger children are able to use an allocentric strategy when aided (Bullens et al., 2010). Overall, these findings suggest that a "developmental window" occurs between 4 and 8 years of age and poses a new and fascinating question regarding whether specific formal training may foster navigational skills in children.

However, compared to other human skills (such as math, reading, writing, and problem solving) that receive formal training during childhood, spatial orientation is not strengthened through specific educational training at school, during childhood or later, with the exception of individuals trained to use this competence at higher levels in a professional context, e.g., aerospace pilots, astronauts, medical surgeons, topographers, or military raiders (Apuzzo, 1996; Verde et al., 2013, 2015, 2016). It is largely accepted that learning produces a brain re-organization that acts as a "brain reserve," increasing the brain's tolerance to disease (Stern, 2012; Colangeli et al., 2016). It has been shown that the brain may be continuously modified by life experiences (Verde et al., 2013, 2015, 2016), even when the brain is affected by neuropathology or when cognitive training enhances the effect of pharmacological therapy (Onder et al., 2005). For this reason, navigational training in pre-schoolers may foster navigational ability and prevent the development of navigational disorders, such as developmental topographical disorientation (DTD: Iaria et al., 2005, 2009; Bianchini et al., 2010). DTD is a developmental disorder for which the etiology is currently unknown; no cerebral damage or psychiatric disorders have been associated with it. Iaria and Barton (2010) showed that DTD is widespread in the population, and the detailed descriptions of healthy individuals who suffer from DTD (refer to Bianchini et al., 2014b; Palermo et al., 2014; Nemmi et al., 2015; Piccardi et al., under revision) appear to suggest that this neurodevelopmental disorder should be monitored from childhood to prevent the persistence of the disorder in adults. The existence of DTD and its different types strongly suggests the importance of introducing navigational training during early infancy. Furthermore, navigational training may be useful for blind children. Research appears to suggest that children with visual impairments tend to rely on egocentric encoding (Ruggiero et al., 2009; Iossifova and Marmolejo-Ramos, 2013). Moreover, visually impaired children show a tendency for narrowing spatial frames by substituting the allocentric space with egocentric or bodily space (Iossifova and Marmolejo-Ramos, 2013). These findings appear to suggest that a lack

of vision may affect allocentric but not egocentric frames of references.

Here, we aimed to investigate the effectiveness of navigational training programme (NTP) to develop spatial orientation skills in 4-year-old children based on Siegel and White's hierarchical model. According to the aforementioned studies, we expected that this early period of life may constitute a developmental window in which it is possible to observe training effects. With this aim, we administered a 12-week NTP and tested the children in both the training and control groups twice (before and after NTP) on navigational tasks that tap into landmark, route and survey knowledge (**Figure 1A**). We hypothesized that the NTP would yield a better performance on highly demanding navigational tasks (i.e., survey knowledge-based tasks; **Figure 1A**) in the training group (TG) than that seen in the control group, which was not exposed to the training. A comparison of these groups enabled us to subtract the normal development of these skills from the improvement produced by the NTP. We expected an earlier acquisition of the ability to form a cognitive map of the environment to be detected only in the group exposed to NTP.

MATERIALS AND METHODOLOGY

Participants

The present study was conducted in a sample of 34 typically developing Italian children (19 M and 15 F) who were recruited from a primary school, the "Istituto Comprensivo di Via Anagni," in Rome (Italy). Ages ranged from 49 to 72 months (mean age = 63.09 months; SD = 2.12 months).

None of the children included in the study had primary visual or hearing impairments, had been diagnosed with a neurological condition or had ever exhibited emotional or behavioral problems. To determine their general cognitive level, all children were administered Raven's Colored Progressive Matrices (Raven, 1986; Belacchi et al., 2008); no difficulty in clear-thinking ability emerged. The study was approved by the local ethics committee of the Psychology Department of "Sapienza" University of Rome in accordance with the Declaration of Helsinki. Written and informed consent was obtained from the parents of each child. The children were subdivided into two groups consisting of 17 participants per group and were comparable in age [$t_{(32)} = 0.08$; $p = 0.93$] and gender [chi-squared = 28.94; $p = 0.62$].

Instruments and Procedure

The children were tested individually in a quiet room in kindergarten. The TG was tested immediately before and after the NTP period at T_0 and T_1, respectively. The control group (CG), which received standard didactics, was tested at T_0 and T_1 without being subjected to a training procedure.

Training Procedure

NTP was conceived to enhance the visuo-spatial abilities that underlie human navigation, such as mental rotation (Piccardi et al., 2017), visuo-spatial and navigational memory (Piccardi and Nori, 2011), navigational planning (Bocchi et al., 2017), spatial orientation, left and right discrimination and spatial representation of the body according to Siegel and White's hierarchical model. Each activity fosters a specific level of the acquisition of navigational knowledge, namely, landmark, route, and survey knowledge. Thus, activities involving landmark and pictorial recognition and perceptual discrimination were thought to foster landmark representation; activities involving the acquisition of directional egocentric information were thought to foster the acquisition of route representation; finally, activities that targeted mental transformation and map-based orientation were thought to foster the acquisition of survey representation (see the description of activities below for details). The activities were administered according to the hierarchical model with increasing levels of difficulty. The NTP was administered at the school and covered 18 sessions (1.5 h each) during a period of 12 weeks (see **Supplementary Figure 1A** for the experimental timeline). The protocol comprised paper-and-pencil and navigational activities (see the detailed description of activities below). The experimenter ascertained that all children played together during navigational activities. Each session was structured to contain both types of activities. Most procedures were inspired by Piccardi (2011) and are subsequently described below according to the level they fostered.

Landmark Knowledge Acquisition

Colored Matrix (Visuo-Spatial Memory)

This task is a paper-and-pencil activity. The children were provided with a set of colored paper-and-pencil matrices (**Supplementary Figure 1B**) presented one by one in the center of a vertically aligned sheet of white paper (A4 format). In each matrix, boxes were only partially filled-in. The children were instructed to observe the colored boxes in the matrix. After 1 min of observation, the children were required to turn the page and fill-in the same boxes in an empty matrix. This task included seven trials, with increasing levels of difficulty.

Large-Scale Memory

This task is a practical spatial game. We developed a larger version of the classic "memory game," the card game in which all cards are placed face down on a surface and only two cards may be turned face up during each turn. The object of the game is to turn over all pairs of matching cards. In the large-scale memory exercise, we used 10 pairs of pictures representing different animals (i.e., sheep, elephant, pig, crocodile), which were manually drawn by one of the authors (LPi) on sheets of white paper (A4 format) and were vertically placed on the floor of a large room covering a surface of 2.5 × 2.5 m. The children were divided into two teams. All participants on each team turned over a pair of sheets in turn. When a participant found the matching animal, the participant took both sheets, and another participant on the same team turned over another pair of sheets. When a participant failed to obtain a match, another participant attempted to obtain a match. The team with the highest number of matching sheets won the game.

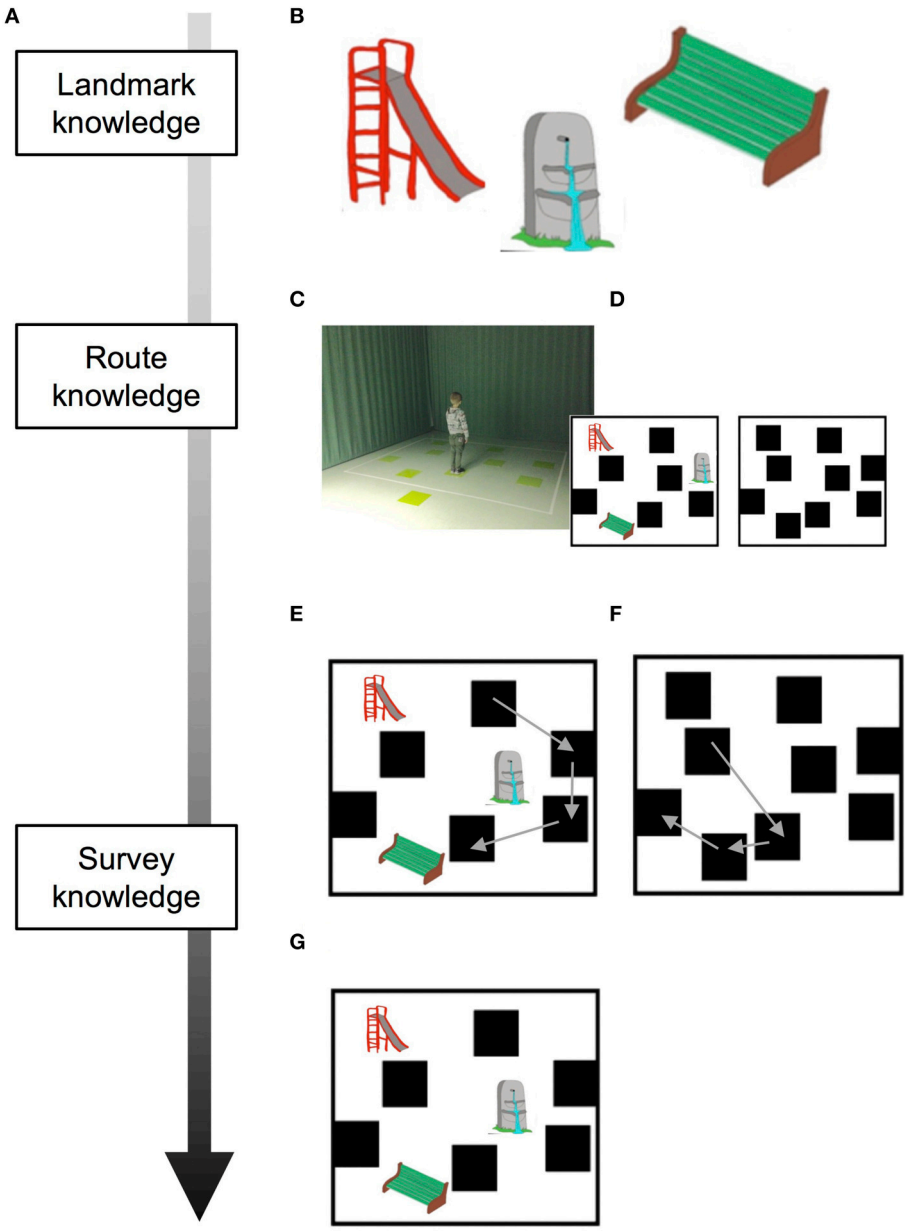

FIGURE 1 | Schematic depiction of tasks and results according to the stages of acquisition of spatial knowledge. **(A)** Siegel and White's model of acquisition of spatial knowledge. **(B)** Landmarks used during WalCT. **(C)** WalCT apparatus; **(D)** Sketch maps of the WalCT with and without landmarks are represented on the bottom-left and -right, respectively. **(E)** Drawing of the pathway in the L-WalCT and **(F)** the WL-WalCT outlines. **(G)** Landmark location on the map. Written informed consent was obtained from both parents of the child represented in the figure for the publication of this study.

Route Knowledge Acquisition

Paper-and-Pencil Labyrinth (Navigational Planning)

This task is a paper-and-pencil activity. The children were provided with a set of pictures, including (a) a subject (a child or an animal), (b) a goal target (e.g., food, water), and (c) a path between the subject and the goal target (**Supplementary Figure 1C**). They were instructed to draw the path the subject had to follow to reach his/her goal. For the first 8 trials, the path was unambiguous, whereas the last 3 trials showed a high level of complexity and some dead ends.

Joystick (Spatial Orientation and Left/Right Discrimination)

This task is a practical spatial game. A leader was selected among the children. All other children were required to place themselves in front of the leader. The leader verbally instructed the participants to move forward, backward, to the left, or to the

right. He/she could give the instruction more or less quickly in order to make it more difficult for them participants to follow his/her instructions. The participants who got the order wrong were out of the game. The last player left became the leader of the following game session.

The Navigator (Spatial Orientation and Left/Right Discrimination)

This task is a practical spatial game. The players were divided into pairs comprising one player guiding (i.e., the guide) and the other navigating blindfolded (i.e., the navigator). At the end of each turn, the players traded places. A guide was chosen, and he/she hid an object. Each guide had to guide his/her blindfolded navigator to be the first to find the hidden object. Only directional (e.g., right, left) and numerical (e.g., 2 steps, 3 steps) cues were allowed. The first pair to find the object could hide it during the following turn and decide which pair should have the blindfolded navigator.

Paths (Spatial Orientation)

This task is a practical spatial game. The children were divided into teams. The teacher built a path, and each team, in turn, had to follow it. The team that completed the path the fastest won the game.

Juggle (Spatial Representation of the Body)

This task is a practical spatial game. Each child received a balloon to juggle. The first children were allowed to juggle with any part of the body. After this initial phase, the children were instructed to juggle with a specific part of the body (e.g., the right hand, the left foot). The child who kept the balloon for the longest time won the game.

Up and Down (Spatial Representation of the Body)

This task is a practical spatial game. The children were divided into two teams. The players on each team formed a line, and the first player of each line had a balloon. At a starting signal, the player with the balloon gave it to the player behind him/her by passing it over his/her head. The second player passed the balloon to the third player by passing it under his/her legs. The third player passed the balloon to the fourth player by passing it over his/her head and so on to the last player in the line. The team whose balloon reached the last player first won the game.

In and Out of the Hoop (Spatial Representation of the Body)

This task is a practical spatial game. The children were divided into two teams. The players of each team formed a line at ∼2 m from the other team. A hoop was given to the first player of the line. At a starting signal, the player stepped into the hoop with his/her foot and lifted it up over his/her head. The player behind him/her grabbed the hoop and did the same, as did the other players until the last player in the line. The team that reached the last player first won the game.

Survey Knowledge Acquisition

Objects' Mental Rotation (Mental Rotation)

This task is a paper-and-pencil activity. The children were provided with a set of 10 sheets where (a) a target flower in the center of the sheet and (b) three test flowers below the target item (**Supplementary Figure 1D**) were depicted. Only one of the three test flowers corresponded to the target. The children were required to find the correct flower among the test flowers.

The Explorers (Spatial Orientation and Navigational Memory)

This task is a practical spatial game. The children were divided into teams or small groups and were invited to navigate through the school (e.g., visiting the garden) for ∼15 min and register as many details (e.g., sounds, odors, objects, and positions) as possible. When they returned to the classroom, they were instructed to draw a map of the path they had taken. They were also required to answer questions regarding the details on their map. The team that drew the best map and answered the most questions won the game.

Testing Procedures

We tested verbal comprehension of spatial locatives using the Test for Reception of Grammar (TROG: Bishop, 1982; Italian version: Chilosi and Cipriani, 1995), which assesses grammatical comprehension from age 4 to adulthood. Each test stimulus is presented in a four-picture, multiple-choice format with lexical and grammatical foils. The grammatical complexity increases consistently from locative structure to active, passive, negative, dative and relative clauses. For experimental purposes, we selected only spatial locative sentences (14 sentences); these sentences included locative topological elements (below/above, up/down, in/out and near/far) and prospective locative elements (in front of/behind, from/to and between). The participants' task was to select the picture that matched a sentence spoken by the examiner; in the case of errors, the sentence was repeated. The score was 0 if the answer was immediately correct, 0.5 if the answer was correct after repetition and 1 if the answer was wrong.

Topographical learning (TL) was assessed using the Walking Corsi Test (WalCT: Piccardi et al., 2008, 2014a,b). The WalCT is a larger version (3 × 2.5 m; scale 1:10) of the Corsi Block Tapping Test (CBT; Corsi, 1972). It has been used for experimental and clinical purposes (Piccardi et al., 2008, 2014a,b; Bianchini et al., 2010, 2014a,b; Nemmi et al., 2013; Palermo et al., 2014) to investigate topographical memory by instructing individuals to reproduce a previously observed pathway. The WalCT is set up in an empty room. It is composed of nine black squares (30 × 30 cm) placed on the floor. This test is scaled to the standard CBT. The starting point is the black square located outside the layout.

In the WalCT, the examiner walks and stops on a series of squares. The subject must walk, reach different locations and reproduce the sequence demonstrated by the examiner. In this study, two aspects of topographical long-term memory were assessed, based on the results of a previous study (Piccardi et al., 2015): TL and topographical delayed recall (TDR) under two different conditions, namely, with landmarks (L-WalCT) and without landmarks (WL-WalCT) (**Figures 1B–D**). The only

difference between the L-WalCT and WL-WalCT conditions was the presence in the L-WalCT of pictures of three landmarks placed on three black squares (**Figure 1B**).

In the TL, the children had to learn a fixed supra-span sequence, which was calculated according to their chronological age by considering the span plus 2 according to the median span sample of Piccardi et al.'s (2014b) study. Specifically, 4-year-old children had to learn a 4-block sequence (because the median span was 1.90 at this age). At each trial, after the examiner presented the sequence, the child was invited to step onto the carpet to reproduce it and step off the carpet when he/she had finished (**Figures 1C–D**). In each trial, the number of correct black squares reproduced in the sequence was calculated for the final score, but no feedback regarding performance correctness was provided. The learning criterion (indicating that learning was achieved) corresponds to three consecutive correct sequence reproductions; if the child did not achieve the learning criterion, the sequence was repeated for a maximum of 18 trials. The learning score was calculated by attributing one point for each square correctly walked until the criterion was achieved; the score corresponding to the correct performance of the remaining trials was added to this score (up to the 18th; maximum score: 72). After 5 min, the TDR was administered. The examiner instructed each participant to reproduce the previously learned four-block sequence in a single attempt. The score represented the number of squares correctly reproduced. TL and TDR may be considered as tapping into *route knowledge* (Siegel and White, 1975).

At the end of the L-WalCT, the children were shown 6 items on an A4 sheet (3 landmarks and 3 distractors). They had to indicate which of the landmarks were present in the L-WalCT (landmark recognition). An individual's score corresponded to the sum of correct responses (maximum score: 6). This task may be considered as tapping into *landmark knowledge* (Siegel and White, 1975). The children were subsequently instructed to place small pictures depicting 3 landmarks on an outline of the WalCT as they experienced them during the administration of the WalCT (landmark location). In this case, an individual's score also corresponded to the sum of correct responses (maximum score: 3) (**Figure 1G**). This task may be considered as tapping into *survey knowledge* (Siegel and White, 1975). At the end of both the L-WalCT and WalCT, the children were also instructed to use a felt-tip marker to retrace the pathways they had learned during the two conditions on the outline of the WalCT. An individual's score corresponded to the sum of the squares correctly identified during the line-tracing (drawing; maximum score: 4) (**Figures 1E,F**). The path line retracing in the drawing of both the L-WalCT and WL-WalCT may be considered the transposition of route knowledge on a map-like/survey representation of the environment and thus an intermediate stage between route- and survey-based knowledge representation (for a schematic depiction of tasks and knowledge representation, see **Figures 1A,E,F**).

Statistical Analysis

For analysis, 2 × 2 mixed factorial ANOVAs with Group (CT and TG) as the between factor and time (T_0 and T_1) as the repeated measures were performed to detect the effect of training on (1) spatial locative comprehension (the number of errors on the TROG), (2) landmark recognition, (3) TL in the L-WalCT, (4) TL in the WL-WalCT, (5) the TDR in the L-WalCT, (6) the TDR in the WL-WalCT, (7) the drawing of the path line on the L-WalCT and (8) on the WL-WalCT outline, and (9) landmark location on the map. The alpha level was set at $p = 0.05$. Eta effect sizes (η2) were computed for main and interaction effects. The benchmarks available to interpret η2 are 0.01–small, 0.06–medium, and 0.14–large (Kittler et al., 2007). However, these benchmarks have not been previously indicated for psychological data (as highlighted by Iossifova and Marmolejo-Ramos, 2013). Therefore, "as η can refer to linear and non-linear relationships, η can be considered a general case in which r is a special example (Rosenthal and Rosnow, 2008). Thus, the generally accepted regression benchmark for effect size r can be used to interpret η: small–0.10, medium–0.30, and large–0.50 (Cohen, 1992)" (p. 2178 in Iossifova and Marmolejo-Ramos, 2013). *Post-hoc* comparisons were performed by applying Bonferroni's correction for multiple comparisons.

RESULTS

The children participated in the training sessions an average of 71.24% (SD = 12.92) of the "maximum" training time.

We identified a main effect of time on spatial locative comprehension as measured by the TROG [$F_{(1, 32)} = 17.89$; $p < 0.001$; Partial Eta Squared = 0.36], with better performances at T_1 (**Table 1**). No other effect was identified on the scores of the TROG.

A main effect of time was also identified for the TL in the L-WalCT [$F_{(1, 32)} = 6.93$; $p = 0.01$; Partial Eta Squared = 0.18] with a similar effect in the WL-WalCT [$F_{(1, 32)} = 4.06$; $p = 0.05$; Partial

TABLE 1 | Means and SDs of the experimental tasks.

Task	T_0		T_1	
	CG	TG	CG	TG
TROG	2.09 (1.63)	2.91 (2.31)	0.618 (1.28)	1.29 (1.25)
LANDMARK KNOWLEDGE-BASED TASK				
Landmark recognition	5.94 (0.24)	5.94 (0.24)	6 (0)	6 (0)
ROUTE KNOWLEDGE-BASED TASKS				
TL, L-WalCT	68.41 (6.29)	69.12 (6.59)	71.29 (1.40)	71.71 (0.85)
TL, WL-WalCT	65.24 (9.47)	68.94 (6.14)	69.71 (2.64)	70.12 (3.82)
TDR, L-WalCT	3.88 (0.49)	4 (0)	3.88 (0.49)	4 (0)
TDR, WL-WalCT	3.76 (0.97)	3.71 (0.77)	4 (0)	4 (0)
ROUTE/SURVEY KNOWLEDGE-BASED TASKS				
Drawing L-WalCT	2.76 (1.52)	2.06 (1.60)	2.94 (1.60)	3.53 (0.87)
Drawing WL-WalCT	2.65 (1.54)	2.35 (1.54)	3.24 (1.35)	3.65 (0.79)
SURVEY KNOWLEDGE-BASED TASK				
Landmark location	2.12 (1.05)	1.76 (1.15)	1.88 (1.27)	2.71 (0.85)

TL, topographical learning; TDR, topographical delayed recall; L, with landmarks; WL, without landmarks; WalCT, Walking Corsi Test; TROG, Test for Reception of Grammar (errors).

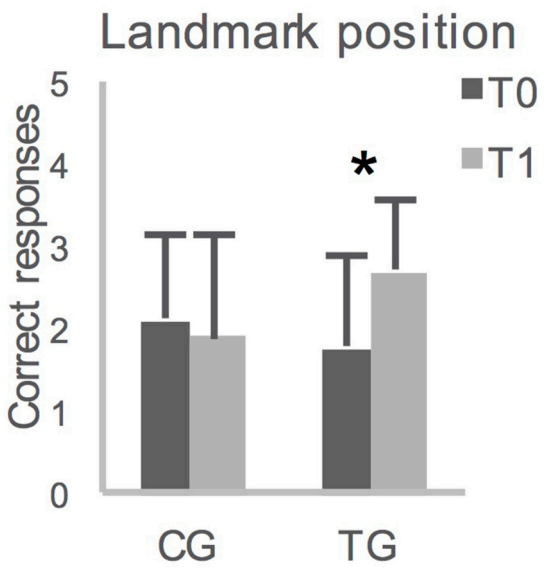

FIGURE 2 | Averaged group performances (and standard deviations) on Landmark location on the map, before (T_0) and after (T_1) 12-weeks. CG, control group; TG, training group; $*p = 0.007$ with Bonferroni's correction for multiple comparisons.

Eta Squared = 0.11]. For both tasks, performances were better at T_1 (**Table 1**).

The ANOVA on performances in the landmark recognition (**Table 1**), as well as the ANOVAs on the TDR in the L-WalCT and the WL-WalCT (**Table 1**), showed no significant effects. The ANOVAs on the drawing of the path line showed a main effect of time both in the L-WalCT [$F_{(1, 32)} = 4.77$; $p = 0.04$; Partial Eta Squared = 0.13] and the WL-WalCT outline [$F_{(1, 32)} = 10.29$; $p < 0.01$; Partial Eta Squared = 0.24], with better performances at T_1 (**Table 1**). Interestingly, the ANOVA on landmark location performances showed a group by time interaction effect [$F_{(1, 32)} = 6.49$; $p = 0.02$; Partial Eta Squared = 0.17]. *Post-hoc* comparisons showed that the two groups significantly differed at T_1 ($p = 0.03$ Bonferroni's correction for multiple comparisons), with the TG performing better than the CG (**Table 1**). Moreover, the children in the TG significantly ameliorated their performances at T_1 compared with that at T_0 (**Figure 2**).

DISCUSSION

We hypothesized that navigational training during childhood may promote an earlier acquisition of high level spatial abilities. Unlike many other abilities, which receive educational training at school during childhood, spatial orientation does not receive systematic training. Nevertheless, high levels of navigational training in adulthood significantly improve spatial orientation ability (Verde et al., 2013, 2015, 2016). The present results show that NTP enhanced the ability to transform egocentric navigational information in a map-like, allocentric representation of the environment in 4- to 5-year old children.

As previously discussed, even if 4-year-old children have basic features of allocentric coding (Negen et al., 2017), children typically master this ability at ∼7 years of age. Spatial orientation abilities are fully functioning by age 10, with an increase in the spontaneous use of allocentric world-centered representation of the environment (Bullens et al., 2010). Similarly, in this study, we found that children (mean 63.09 ± 2.12 months) who underwent NTP were more proficient in locating landmarks on a map than their peers who did not receive undergo NTP. This result suggests that NTP yields an earlier development of an allocentric world-centered representation of the environment. To locate landmarks on a map, children must translate egocentric tri-dimensional information regarding the environment they experience in an allocentric bi-dimensional representation. This operation is similar to a "cognitive map," that is, a stable mental spatial representation independent of perception (Tolman, 1948; Boccia et al., 2017). When required to locate landmarks on a map, children must retrieve navigational information regarding their position from memory and convert it into a map-like representation. As a result of NTP, the children developed the ability to use allocentric coding earlier, and they could pinpoint a location in relation to other locations rather than using egocentric coding. The use of allocentric coding requires an individual to generate, maintain, inspect and transform an image in the mind. These aspects of mental imagery (Kosslyn, 1980) are pivotal to human navigation (Pazzaglia and De Beni, 2006; Palermo et al., 2012; Piccardi et al., 2017). The NTP encompassed activities involving mental rotation (i.e., mental transformation) and visuo-spatial memory (i.e., maintaining an online mental image), as well as activities focused on spatial orientation, left/right discrimination, navigational memory, and planning. The finding that the TG achieved the ability to use an allocentric coding earlier than the CG suggests that a formal training of visuo-spatial abilities that underlie human navigation (such as mental rotation, visualization, or navigational problem solving) improves the ability to transform egocentric representation into an allocentric representation.

Interestingly, our data suggest that the development of spatial locative comprehension and mastery of route knowledge-based tasks (i.e., L-WalCT and WL-WalCT) and the path-line drawing on a map (i.e., Drawing L-WalCT and Drawing WL-WalCT) spontaneously improved from T_0 to T_1 in the CG as well. In contrast, landmark location on the map is the only task significantly improved by NTP. Route knowledge-based tasks require only an egocentric frame of reference, which may also be used during a task that requires drawing a path. Individuals who have not fully developed an allocentric strategy may trace the line of the path by simply repeating the egocentric path they learned. Instead, when individuals are asked to locate landmarks on a map, they must use both egocentric and allocentric information. Thus, even if performances on route knowledge-based tasks spontaneously increase in 4- to 5-year-old children, the NTP allowed the children in the TG to move to the next developmental step, that is, to form and use an allocentric representation of space.

The TDR of a pathway (in both the L-WalCT and WL-WalCT) did not show an effect of time or training. It must be noted that performances on these tasks were subject to a ceiling effect by

the T_0. This result suggests that once a pathway is learned, its mental representation is stable and long-lasting. This finding is also in line with the performances of young adults in the same task (Piccardi et al., 2008, 2011, 2013).

Moreover, in landmark recognition, we did not identify an effect of time or NTP. We assume that this step is fully developed in 4- to 5-year-old children. It is a first step of environmental knowledge (Siegel and White, 1975) and includes the identification of landmarks (Thorndyke and Hayes-Roth, 1982). This finding is also in line with that of a previous study demonstrating that young individuals unfamiliar with the environment were able to identify landmarks but could not place them on a map (Nori and Piccardi, 2011). Landmark recognition is the first step in the familiarization process with a new environment (Nori and Piccardi, 2011). Interestingly, as opposed to landmark recognition, we found that children improve on the TL of the L-WalCT between T_0 and T_1. This result suggests that even if landmark recognition was fully achieved, the ability to integrate landmark-based knowledge with route-based knowledge in the L-WalCT is still developing at age 5. It must be noted that integrating landmark-based knowledge with route-based knowledge is a next step in the normal development of navigational skills. Navigation is typically expected to be easier when landmarks are available in the environment (Nico et al., 2008). Moreover, environments enriched in landmarks assist 5- to 7-year-old children in orienting themselves (Hermer-Vazquez et al., 2001), with an automatic shift toward a landmark-based strategy that results in a significant improvement in performances. This observation also holds in adulthood (Nico et al., 2008; Piccardi, 2009). However, a 20-year-old woman who has never been able to orient herself within the environment as a result of a congenital brain malformation showed the worst performance in a way-finding task when landmarks were available, despite preserved landmark identification (Iaria et al., 2005). This observation also holds in the case of acquired deficits, specifically in right-brain-damaged patients with hemineglect, who did not improve their performances when landmarks were available in the environment (Nico et al., 2008), even if patient performances may be dissociated (Pizzamiglio et al., 1998; Piccardi, 2009). Thus, our data suggest that this developmental step, which may be selectively prevented by a congenital malformation and damaged in acquired brain lesions, is still developing in 4- to 5-year-old children, despite evidence from typical development that demonstrates an earlier acquisition of this step at ∼24 months of age (Hermer and Spelke, 1994, 1996).

In general, our results suggest that the normal development of spatial orientation abilities may be considered to follow a continuum rather than a serial organization of navigational mechanisms, which is consistent with previous neuropsychological evidence (e.g., Bianchini et al., 2010, 2014a; Palermo et al., 2014). In light of Siegel and White's cumulative model (1975), we speculate that intermediate stages exist between landmark- and route-based knowledge and between route- and survey-based knowledge. Within these intermediate stages, information coded in different formats is integrated and results in improved performance. Within this framework, even if in the presence of spontaneous improvements in integrating landmark- and route-based knowledge (performance on L-WalCT) and integrating route- and survey-based knowledge (path line drawing), NTP specifically improves performances at the highest level of navigational task. Specifically, the allocentric representation of environmental places arises from the transformation of an egocentric frame of reference. Interestingly, NTP may be useful not only in preventing developmental navigational deficits but also in helping children with visual impairments. As demonstrated by Iossifova and Marmolejo-Ramos (2013), blind children show a difficulty in the use of allocentric coding that may be addressed with specific training, such as an adapted version of NTP for visually impaired children.

Although our results are novel and the future applications in education are interesting, the present study has several weaknesses. In particular, the sample size is small, which represents a potential limitation in the statistical approach that may be adopted. In a future study, we must implement non-parametric or robust approaches to data analyses by increasing the sample size and enrolling participants at different ages of development. Moreover, further studies are needed to understand the long-term effects of the improvement identified in the TG and whether formal training later in life has the same effect on spatial skills. The consequences of NTP in preventing the development of navigational disorders should also be investigated in future studies.

In conclusion, our findings support the idea that the inclusion of formal training of spatial orientation ability during childhood may result in enhanced navigational abilities, particularly for the highest level navigational task.

AUTHOR CONTRIBUTIONS

LPi, CG, and SD designed and conceived the study. MR and FV collected data. MB, AT, and LPa analyzed data and MB wrote a first draft of the manuscript which has been further revised by all the authors. All the authors contributed to the discussion of the results.

ACKNOWLEDGMENTS

The authors thank the "Istituto Comprensivo di Via Anagni" in Rome for their cooperation and interest in this research. In particular, the authors thank the School's Director, Dr. Maura Frasca, for her interest in this project, as well as Dr. Chiara Bellagamba and Dr. Veronica Sacco for their help with the children during NTP. We also acknowledge the children who participated in the study and their families, who provided their consent.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2017.00574/full#supplementary-material

Supplementary Figure 1 | (A) Experimental timeline; **(B)** An example of an item of the Colored Matrix. The children were instructed to observe the colored boxes in the matrix (up). After 1min of observation, the children were required to turn the page and fill-in the same boxes in an empty matrix (down); **(C)** An example of an item of an unambiguous Paper-and-Pencil Labyrinth. The children were required to help the bee to reach flowers; **(D)** An example of an item of the Objects' Mental Rotation. The children's task was to observe the target flower in the center of the sheet and to identify the only correct one of the three rotated flowers corresponding to the target.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 17 October 2017
doi: 10.3389/fphar.2017.00725

Inactivation of the Prelimbic Cortex Impairs the Context-Induced Reinstatement of Ethanol Seeking

*Paola Palombo[1,2], Rodrigo M. Leao[3], Paula C. Bianchi[1,2], Paulo E. C. de Oliveira[1], Cleopatra da Silva Planeta[1,2] and Fábio C. Cruz[4]**

[1] Laboratory of Pharmacology, School of Pharmaceutical Sciences, Universidade Estadual Paulista, São Paulo, Brazil, [2] Joint Graduate Program in Physiological Sciences UFSCar/UNESP, São Carlos, Brazil, [3] Departamento de Biorregulação, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Brazil, [4] Department of Pharmacology, São Paulo Federal University, São Paulo, Brazil

Evidence indicates that drug relapse in humans is often provoked by exposure to the self-administered drug-associated context. An animal model called "ABA renewal procedure" has been used to study the context-induced relapse to drug seeking. Here, we reported a new and feasible training procedure for the ABA renewal method to explore the role of the prelimbic cortex in context-induced relapse to ethanol seeking. By using a saccharin fading technique, we trained rats to self-administer ethanol (10%). The drug delivery was paired with a discrete tone-light cue. Lever pressing was subsequently extinguished in a non-drug-associated context in the presence of the discrete cue. Rats were subsequently tested for reinstatement in contexts A or B, under extinction conditions. Ethanol-associated context induced the reinstatement of ethanol seeking and increased the expression of Fos in the prelimbic cortex. The rate of neural activation in the prelimbic cortex was 3.4% in the extinction context B and 7.7% in the drug-associated context A, as evidenced by double-labeling of Fos and the neuron-specific protein NeuN. The reversible inactivation of the neural activity in the prelimbic cortex with gamma-Aminobutyric acid (GABA) receptor agonists (muscimol + baclofen) attenuated the context-induced reinstatement of ethanol self-administration. These results demonstrated that the neuronal activation of the prelimbic cortex is involved in the context-induced reinstatement of ethanol seeking.

Keywords: prelimbic, pharmacologic inactivation, context, reinstatement, ethanol

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Bruce Thomas Hope, National Institute on Drug Abuse, United States
Cristiano Chiamulera, University of Verona, Italy

***Correspondence:**
Fábio C. Cruz
ccruzfabio@yahoo.com.br

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *26 April 2017*
Accepted: *27 September 2017*
Published: *17 October 2017*

Citation:
Palombo P, Leao RM, Bianchi PC, de Oliveira PEC, Planeta CS and Cruz FC (2017) Inactivation of the Prelimbic Cortex Impairs the Context-Induced Reinstatement of Ethanol Seeking. Front. Pharmacol. 8:725. doi: 10.3389/fphar.2017.00725

INTRODUCTION

Ethanol is the most commonly used addictive substance worldwide (UNODC, 2016). The harmful use of ethanol is responsible for 3.3 million deaths each year (UNODC, 2016). Relapse represents a prevalent and significant problem in ethanol addiction. Indeed, given the high rate of recidivism in alcoholism, relapse is clearly a major impediment to treatment efforts of this disorder (Fox et al., 2008; Sinha, 2009; Becker and Koob, 2016).

Evidence indicates that drug relapse in humans is often provoked by exposure to the self-administered drug-associated context (O'Brien et al., 1990; Gauggel et al., 2010). In this regard, clinical reports and laboratory studies have shown that factors associated with ethanol consumption may induce relapse in humans (O'Brien et al., 1990; Kirk and de Wit, 2000;

Litt and Cooney, 2000). For instance, abstinent alcoholics have reported that specific environmental cues elicit craving for ethanol (McCusker and Brown, 1990; Litt et al., 2000; Gauggel et al., 2010). An animal model known as "ABA renewal procedure" has been used to study the context-induced relapse to drug seeking (Bouton et al., 2006; Crombag et al., 2008; Bouton, 2011, 2014; Bouton et al., 2014; Bouton and Schepers, 2015). In this animal model, rats are trained to self-administer the drug in a context (context A); during the training, drug infusions are paired with a discrete tone-light cue. The lever pressing is subsequently extinguished in a non-drug-associated context (context B) in the presence of the discrete cue. The rats are subsequently tested for reinstatement in the drug-associated context under extinction conditions (Crombag et al., 2008; Lasseter et al., 2010; Marchant et al., 2013). Since it was first demonstrated by Burattini et al. (2006), context-induced ethanol seeking has been replicated in several studies (Bossert et al., 2013; Marchant et al., 2014; Willcocks and McNally, 2014).

Studies have demonstrated the participation of cortical areas in both relapse and extinction of drug seeking (Bossert et al., 2012; Willcocks and McNally, 2013; Marchant et al., 2015). Specifically, the prelimbic cortex is associated with reinstatement of drug seeking, while the infralimbic cortex is implicated in the extinction of drug seeking (Peters et al., 2009; Van den Oever et al., 2010). In this regard, it was found that inactivation of the prelimbic cortex (using the sodium channel blocker tetrodotoxin-TTX) impaired the context-induced cocaine seeking (Fuchs et al., 2005). Moreover, the reversible inactivation (using muscimol + baclofen, gamma-aminobutyric acid (GABA) a + GABAb agonists, respectively) of the infralimbic cortex impaired the extinction of cocaine self-administration (Muigg et al., 2008). Similarly, Willcocks and McNally (2013) reported that the reversible inactivation of prelimbic cortex by muscimol + baclofen decreases context-induced reinstatement of alcoholic beer-seeking.

However, the modulation of extinction and seeking behavior by infralimbic *versus* prelimbic cortex is supported by numerous studies (Rogers et al., 2008; Koya et al., 2009a; Rocha and Kalivas, 2010; Warren et al., 2016). While some studies reported a role of the prelimbic cortex in drug seeking behavior (McFarland and Kalivas, 2001; Kalivas and McFarland, 2003; Peters et al., 2009; Calu et al., 2013), other studies have indicated that the inactivation of the prelimbic areas does not attenuate the reinstatement of drug seeking (Koya et al., 2009a; Bossert et al., 2011; Li et al., 2015a). Although the infralimbic cortex has been implicated in the extinction of drug seeking (Peters et al., 2009; Warren et al., 2016), many studies have demonstrated that the inactivation of the ventral medial prefrontal cortex (brain area that comprises the infralimbic cortex) has no effect on this behavior (Rogers et al., 2008; Koya et al., 2009a; Rocha and Kalivas, 2010; Warren et al., 2016). Recently, it was demonstrated that the ventral medial prefrontal cortex encodes neuronal ensembles related to both food reward and extinction memories (Rhodes and Killcross, 2004, 2007; Ishikawa et al., 2008; Peters et al., 2008; Warren et al., 2016).

Here, we used Fos immunohistochemistry and the mixture of muscimol and baclofen in the activation procedure to examine whether the context-induced reinstatement of ethanol seeking is mediated by the prelimbic cortex.

MATERIALS AND METHODS

Animals

We used rats (male Long-Evans, 350–450 g; $n = 86$), obtained from the animal breeding facility of the São Paulo State University-UNESP. Groups of four animals were housed in plastic cages (32 × 40 × 16 cm) with unrestricted access to food and water. Rats were continuously maintained on a reversed light/dark cycle (12 h/12 h, lights off at 07:00 a.m.) in a room with controlled temperature (23 ± 2°C). All experiments were performed during the dark phase. The experimental protocol was approved by the Ethical Committee for Use of Animal of Physical Institute of São Carlos, São Paulo University (Protocol #01/2015) and were conducted according to the ethics principles of the Conselho Nacional de Controle de Experimentação Animal (CONCEA).

Twenty-one rats were excluded from the study: 14 for poor training (<10 reinforcements/day) or because they did not reach the extinction criterion (<25 responses/day), four for misplaced cannulae, and three because they lost their head caps.

Apparatus

Standard Med Associates (St. Albans, VT, United States) self-administration chambers were used in all experiments. Two different contexts were set up as described in Cruz et al. (2014). Context A corresponded to the ethanol paired context and context B corresponded to the non-drug extinction context.

Drugs

The following compounds were used: Ethanol 96% (Synth); flunixine-meglumine (Schering-Plough); streptomycin and penicillin polyantibiotic (Fort Dodge); tribromoethanol (Sigma–Aldrich); baclofen (Tocris Bioscience); muscimol (Tocris Bioscience); and saccharin (Sigma).

Experimental Design

We used a protocol adapted from Willcocks and McNally (2013). By using a saccharin fading technique, we trained rats to self-administer ethanol (10%) over 24 days. The drug delivery was paired with a discrete tone-light cue. Lever pressing was subsequently extinguished in a non-drug-associated context in the presence of the discrete cue during 8 days. The rats were subsequently tested for reinstatement in context A or context B under extinction conditions (**Figure 1**).

Ethanol Self-administration Training

Initially, the rats had free access to ethanol (10% w/v) and water for 3 days consecutively in their homecages, to habituate them to the ethanol taste, as previously described by Leão et al. (2015). Subsequently, they were trained to self-administrate 10% ethanol using a saccharin fading procedure. Twenty-four sessions were performed during the training phase: Four sessions where 0.1 ml of 0.2% saccharin solution was delivered, followed for two

sessions where an active lever press resulted in the delivery of 0.1 ml of 0.2% saccharin-plus-10% ethanol (w/v). Subsequently, we performed two sessions where an active lever press resulted in the delivery of 0.1 ml of 0.05% saccharin-plus-10% ethanol (w/v), followed by six sessions where only 10% ethanol (w/v) was delivered. During these phases of the training in context A, saccharin and/or ethanol reinforcements were earned under a fixed ratio 1 (FR1) with a 20-s timeout reinforcement schedule and paired with a compound tone (2,900 Hz; 20 dB above background) and light (7.5 W white light) cue for 2.3 s. Following the FR1 session, we trained rats on a variable-interval 30-s (VI-30) schedule of reinforcement for 10 sessions (Marchant et al., 2016). During the VI-30 sessions, a 10% ethanol (w/v) delivery was available after an active lever press at pseudo-random intervals (1–59 s) after the preceding ethanol delivery. The ethanol deliveries were also paired with a compound tone (2,900 Hz; 20 dB above background) and light (7.5 W white light) cue for 2.3 s.

The initiation of each training session was signaled by the illumination of the house-light and the insertion of the active lever into the chamber. Inactive lever presses had no programmed consequences. All training sessions were performed for 1 h each.

After the end of the last VI-30 session, blood samples (50 μl) were collected from the tip of the tail of each rat. The blood samples were analyzed by an enzymatic system (AM1 Analyzer, Analox Instruments Ltd, London, United Kingdom) on the basis of the measurement of oxygen consumption in the ethanol-acetaldehyde reaction. This procedure was performed to check if all the delivered ethanol was consumed by the animals.

Extinction of Ethanol Self-administration

Extinction was performed in a non-drug-associated context (context B) in the presence of the same discrete cue described above, but the responses on the previously active lever were reinforced by ethanol delivery. The ethanol self-administration behavior was considered extinct when the rats met the extinction criterion of <15 presses per 1 h session. A minimum of eight extinction sessions were performed.

Test for Context-Induced Reinstatement

We tested the rats for ethanol seeking (active lever presses under extinction conditions) in 30-min sessions in contexts A or B under extinction parameters (VI-30 schedule of reinforcement), in which an active lever press was not reinforced by ethanol delivery (Cruz et al., 2014).

Implantation of Intracranial Cannulas

After the rats were anesthetized with tribromoethanol (250 mg/kg; intraperitoneal injection [i.p.]), permanent guide cannulas (23-gauge, Master-One Ribeirão Preto, São Paulo, Brazil) were implanted bilaterally 1 mm above the prelimbic cortex. We used the stereotaxic coordinates according to Paxinos and Watson (2005) and according to Willcocks and McNally (2013). The nose bar was set at −3.3 mm and the coordinates for the prelimbic cortex were as follows: anteroposterior (AP) +3.0 mm, medial lateral (ML) ± 1.5 mm (10° angle), and dorsal ventral (DV) −3.0 mm (**Figure 5C**). After the surgery, the rats were treated with a streptomycin and penicillin polyantibiotic formulation (0.27 mg/kg, intramuscular injection [i.m.]; Pentabiotico, Fort Dodge, Campinas, São Paulo, Brazil) to prevent infections, and received the non-steroidal anti-inflammatory drug flunixine-meglumine (0.025 mg/kg, i.m.; Banamine, Schering-Plough, Cotia, São Paulo, Brazil) for post-operative analgesia.

Intracranial Injections and Histology

Fifteen minutes prior to the test, bilateral injections of saline or muscimol (0.03 nmol/0.5 μl/side) + baclofen (0.3 nmol/0.5 μl/side) (Tocris Bioscience) dissolved in saline were performed in the prelimbic cortex. The doses were based on previous studies (McFarland and Kalivas, 2001; Bossert et al., 2011; Cruz et al., 2014). The intracranial injections were administered using a syringe pump (Harvard Apparatus, Holliston, MA, United States) and 10 μl Hamilton syringes that were attached via polyethylene 50 tubing to 30-gauge injectors (Plastics One). Muscimol + Baclofen or saline were injected over 1 min and the injectors were left in place for 1 min. At the end of the study, the rats were injected with an overdose of tribromoethanol (500 mg/kg, i.p.). Subsequently, their brains were removed, frozen, and sectioned coronally at 40 μm using a cryostat. All sections containing the cannula tracts were collected, stained for cresyl violet, and coverslipped with Permount (Sigma).

Experiments

Experiment 1: Context-Induced Reinstatement of Ethanol Seeking

In the test group (A-B-A), ethanol self-administration was trained in context A, extinction training in context B (1 h per day), and reinstatement test in context A (30 min). In the control group (A-B-B), ethanol self-administration was trained in context A, extinction training in context B (1 h per day), and reinstatement test in context B (30 min). A total of 34 animals were used. The experimental design was based on Cruz et al. (2014).

Experiment 2: Prelimbic Cortex Neuronal Activation after Context-Induced Reinstatement of Ethanol Seeking

We used immunohistochemistry to characterize the involvement of the prelimbic cortex in the context-induced reinstatement of ethanol operant self-administration.

At the end of the reinstatement test, the rats used in Experiment 1, were anesthetized with tribromoethanol (250 mg/kg, i.p.) and perfused with 100 ml phosphate-buffered saline (PBS) followed by 400 ml 4% paraformaldehyde.

The brains were post-fixed in 4% paraformaldehyde for 90 min and transferred to 30% sucrose in PBS at 4°C for 2–3 days. Brains were frozen in powdered dry ice and kept at −80°C until sectioning. Coronal sections were cut at 40 μm. Free-floating sections were washed three times in PBS, blocked with 3% normal goat serum (NGS) in PBS with 0.25% Triton X-100 (PBS-Tx), and incubated for 24 h at 4°C with anti-Fos antibody (1:4000, sc-52; Santa Cruz Biotechnology) diluted in blocking solution. After further washing with PBS, sections were incubated for

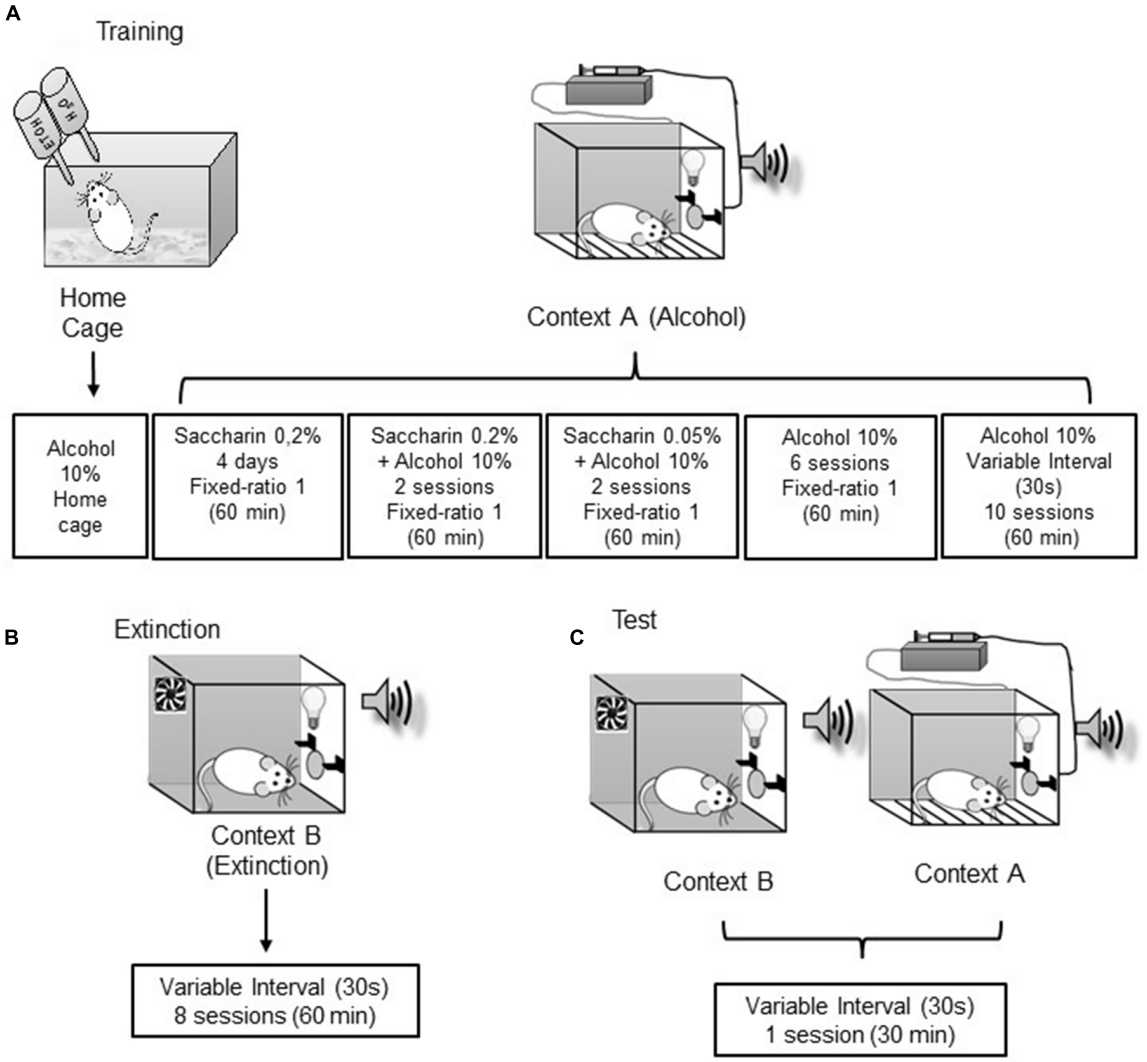

FIGURE 1 | *Schematic representation of the experimental design*. The protocol was adapted from Willcocks and McNally (2013). Briefly, rats were given access to 10% ethanol in their home cages. Using a saccharin fading technique, rats are trained to self-administer 10% ethanol in context A. Subsequently, the lever pressing in the presence of the discrete cue was extinguished in context B. The context-induced reinstatement of drug seeking was assessed by re-exposing the rats to context A or B under extinction conditions. See Methods for more details. **(A)** Training phase; **(B)** Extinction phase; **(C)** Test day.

2 h with biotinylated goat anti-rabbit secondary antibody (1:400; Vector Laboratories) in PBS-Tx and 1% NGS. After washing in PBS, sections were incubated for 1 h in avidin-biotin-peroxidase complex (ABC Elite kit, PK-6100; Vector Laboratories) in PBS containing 0.5% Triton X-100. Finally, sections were washed in PBS and developed in 3,3′-diaminobenzidine for approximately 3 min, transferred into PBS, and mounted onto chrome alum-gelatin-coated slides. Once dry, the slides were dehydrated through a graded series of alcohol and cleared with xylol (LabSynth, SP, Brazil) before coverslipping with Permount (Sigma-Aldrich, St. Louis, MA, United States).

Bright-field images of Fos immunoreactivity in the prelimbic cortex were captured by using a CCD camera (Coolsnap Photometrics, Roper Scientific Inc.) and QimagingExi Aqua attached to a Zeiss Axioskop 2 microscope. Images for counting the labeled cells were captured at 100 × magnification. Labeled cells from 3–4 hemispheres per rat were automatically counted using IPLab software for Macintosh, version 3.9.4 r5 (Scanalytics Inc.) and iVision for Macintosh, version 4.0.15 (Biovision). Each rat was considered as one sample, and the cell counts from all the images of each rat were averaged for statistical comparisons (Cruz et al., 2014).

Experiment 3: Quantification of the Activated Cortex Neurons Using Double-Labeling Immunofluorescence

We used double-labeling immunofluorescence to characterize the neuronal activation of the prelimbic cortex during the reinstatement tests. For these experiments, we used four animals from each group. All rats were perfused with 4% paraformaldehyde 90 min after the beginning of the

reinstatement test. The dissected brains were processed as described above and were kept at −80°C until sectioning. Coronal sections were cut between bregma +2.5 mm and +3.7 mm (Paxinos and Watson, 2005).

For these assays, we used sections obtained from a subset of the brains used in the previous Fos immunohistochemistry assays ($n = 4$ rats from each group). The proportion of all prelimbic cortical neurons expressing Fos during the reinstatement test were determined by double-labeling for Fos and the neuron-specific protein NeuN. For Fos + NeuN labeling, sections were washed three times in Tris-buffered saline (TBS) and permeabilized for 30 min in TBS with 0.2% Triton X-100. Sections were incubated in primary antibodies diluted in TBS with 0.2% Triton X-100 for 24 h on a shaker at 4°C. Primary antibodies were rabbit anti-Fos (1:400, sc-52; Santa Cruz) and mouse anti-NeuN (1:2000, MAB37; EMD Millipore). The sections were further washed three times in TBS and incubated with secondary fluorescent antibodies diluted in TBS with 0.2% Triton X-100 for 2 h on a shaker at room temperature. The secondary antibodies were Alexa Fluor 488-labeled donkey anti-rabbit (1:200, A-10042; Invitrogen) and Alexa Fluor 560 donkey anti-mouse (1:2000, A-10238; Invitrogen) to label Fos and NeuN, respectively. After labeling, sections were washed in TBS, mounted onto chrome alum-gelatin-coated slides, and coverslipped with VectaShield hard-set mounting media. All fluorescent images of the prelimbic cortex were captured by using a CCD camera (Coolsnap Photometrics, Roper Scientific) attached to a Zeiss Axioskop 2 microscope. Images for the co-localization of Fos and NeuN were captured at 200× magnification. The number of Fos-labeled and double-labeled cells from the prelimbic cortex of one section per rat were counted using iVision for Macintosh, version 4.0.15 (Biovision Technologies).

We determined the proportion of all prelimbic cortical neurons expressing Fos (i.e., $Fos^{+}NeuN^{+}$ cells) during the reinstatement test as described in Cruz et al. (2014).

Experiment 4: Effect of the Pharmacological Inactivation of the Prelimbic Cortex on the Context-Induced Reinstatement of Ethanol Seeking

Thirty-one Long-Evans rats were anesthetized and implanted with permanent bilateral guide cannulas into the prelimbic cortex as described above. Subsequently, rats underwent ethanol self-administration training and extinction as described above. On the test day, the rats received bilateral injections of either muscimol (0.03 nmol/0.5 μl/side) + baclofen (0.3 nmol/0.5 μl/side) (TocrisBioscience) dissolved in sterile saline 0.9% or saline 0.9% alone, 15 min prior to the reinstatement test as described above. The number of rats per group was as follows: vehicle context B, $n = 8$; baclofen + muscimol context B, $n = 6$, vehicle context A, $n = 9$; and baclofen + muscimol context A, $n = 8$. To rule out the possibility that the effect of baclofen + muscimol on the test day was due to motor deficits, 18 rats were trained after the completion of this experiment to lever-press for 0.2% of saccharin under an FR1 and 20-s timeout reinforcement schedule for five 60-min sessions. Subsequently, we assessed the effect of vehicle or baclofen + muscimol injections into the prelimbic cortex on the saccharin-maintained responding in a 30-min session. At the end of the test session, the rats were deeply anesthetized with tribromoethanol (500 mg/kg) and perfused with 100 ml PBS followed by 400 ml paraformaldehyde (4%). The brains were post-fixed in 4% paraformaldehyde for 90 min and transferred to 30% sucrose in PBS at 4°C for 2–3 days. The brains were frozen in powdered dry ice and kept at −80°C until sectioning.

Statistical Analyses

All statistical analyses were performed by using Statistic, StatSoft. The data were analyzed by analysis of variance (ANOVA); Newman–Keuls test was used for *post hoc* analyses when the ANOVA indicated significant main or interaction effects ($p < 0.05$).

RESULTS

Experiments 1–4: Training and Extinction

Figures 2A,B depicts the mean (±standard error of the mean [SEM]) the number of ethanol reinforcements and presses on the active and inactive levers during the training phase in context A in all experiments. The rats displayed a consistent ethanol self-administration, as indicated by the increase in the number of infusions and active lever presses over the training sessions. **Figures 2C,D** depicts the mean (±SEM) number of lever presses on the previously active and inactive levers during the first eight extinction sessions in context B. As expected, the active lever presses decreased over time. **Figure 3** shows the correlation between the number of reinforcements achieved during the last training session and the blood ethanol levels, indicating a significant correlation ($r = 0.5735$, $p < 0.01$; **Figure 3**).

For experiments 1–3, on the test day, we assessed the context-induced reinstatement of ethanol seeking by assessing the non-reinforced lever presses in context A *versus* context B (**Figure 4A**). The exposure to context A, but not context B, increased the non-reinforced active lever pressing. The results indicated a significant interaction between context (A and B) and lever (active and inactive) ($F_{1,64} = 19.89$, $p < 0.0001$).

Experiment 2: Context-Induced Reinstatement of Ethanol Seeking Is Associated with Increased Fos Expression in the Prelimbic Cortex, But Not in the Infralimbic and Cingulate Cortex

In Experiment 2, we determined whether the context-induced reinstatement of ethanol seeking was associated with increased Fos-immunoreactive nuclei (Fos-IR) in the prelimbic, infralimbic, and cingulate cortex (**Figure 4C**). We analyzed the Fos expression by using the between-subject factor of context (contexts A and B) and the within-subject factor of the cortical region (cingulate, infralimbic, and prelimbic).

Exposure to context A increased the number of Fos-IR nuclei in the prelimbic cortex, but not in the infralimbic

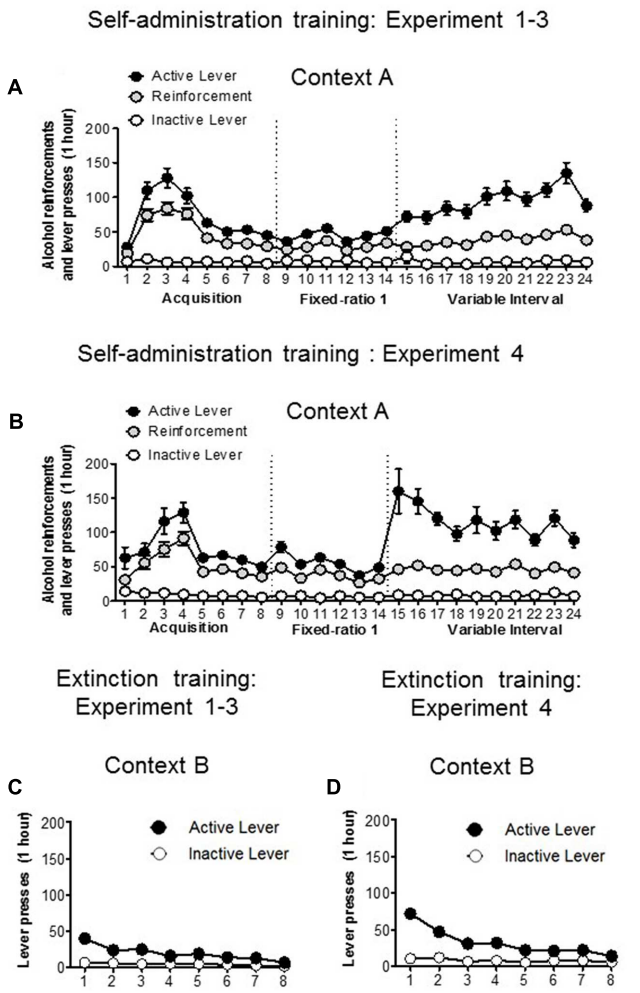

FIGURE 2 | Training and extinction of ethanol reinforcement responding. **(A,B)** Rats were trained to self-administer ethanol over 24 days. Data depict the mean (±standard error of the mean [SEM]) number of infusions, and active and inactive lever presses during ethanol self-administration training in context A in Experiments 1–3 ($n = 34$) and experiment 4 ($n = 31$). **(C,D)** Mean (±SEM) number of presses on the active lever and inactive lever during eight extinction sessions conducted in context B in Experiments 1–4.

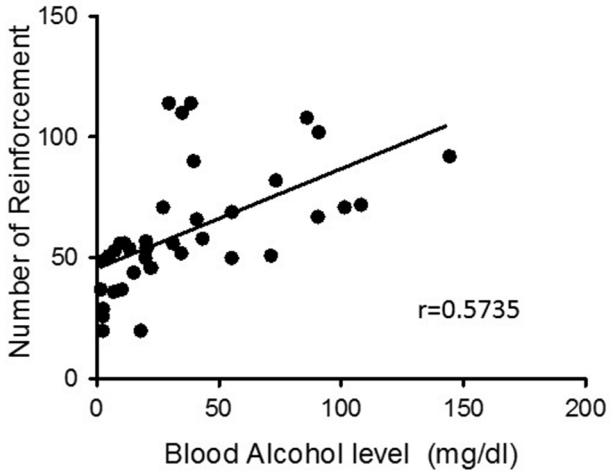

FIGURE 3 | Blood ethanol determination. Immediately after the end of the last VI-30 session, blood samples (50 μl) were collected from the tip of the tail of each rat. The graph depicts the correlation plots of the number of reinforcements achieved during the last training session as a function of the blood ethanol level ($p < 0.01$).

and cingulate cortical regions (**Figure 4B**). The results in the prelimbic cortex further demonstrated a significant main effect of the context ($F_{1,17} = 6.24$; $p < 0.01$). The number of Fos-IR nuclei was higher in context A than in context B ($p < 0.01$).

Experiment 3: Quantification of Activated Cortex Neurons Using Double-Labeling Immunofluorescence

In Experiment 3, we used double-labeling immunofluorescence (**Figures 4E–G**) to determine the percentage of Fos-expressing neurons in sections obtained from a subset of the brains used in Experiment 1 ($n = 4$ rats per group). In the prelimbic cortex, the percentage of activated neurons was 3.4 ± 0.1% and 7.74 ± 0.3% of all neurons following the exposure to context B and context A, respectively. The percentage of activated neurons observed in the infralimbic cortex was 2.5 ± 1.3% and 3.16 ± 0.62% following the exposure to context B and context A, respectively. The percentage of activated neurons in the cingulated cortex was 1.1 ± 0.1% and 3.96 ± 1.31% following exposure to contexts B and A, respectively (**Figure 4D**). The results in the prelimbic cortex further demonstrated a significant main effect of the context ($F_{1,7} = 24.41$; $p < 0.0001$). The percentage of Fos-IR nuclei was higher in context A than in context B ($p < 0.001$).

Experiment 4: Muscimol + Baclofen Inactivation of the Prelimbic Cortex Decreased the Context-Induced Reinstatement of Ethanol Seeking

In this experiment, we used a reversibly inactivating procedure by applying muscimol + baclofen (McFarland and Kalivas, 2001; Bossert et al., 2011; Bossert et al., 2012; Cruz et al., 2014) to determine the role of the prelimbic cortex in the context-induced reinstatement of ethanol seeking. The results of this experiment indicated a significant interaction among context (A, B), lever (active, inactive), and drug (vehicle, muscimol + baclofen), $F_{1,58} = 5.15$; $p < 0.05$.

For active lever, the two-way ANOVA indicated significant interaction between context (A, B) and drug (vehicle, muscimol + baclofen) factors, $F_{1,29} = 5.50$; $p < 0.001$. The *post hoc* statistical analysis indicated that the muscimol + baclofen injections into the prelimbic cortex attenuated the active lever pressing in context A ($p < 0.001$), but not in context B (**Figure 5A**).

For inactive lever, none interaction was observed between context (A, B) and drug (vehicle, muscimol + baclofen) factors, $F_{1,29} = 0.20$; $p > 0.05$ (**Figure 5B**).

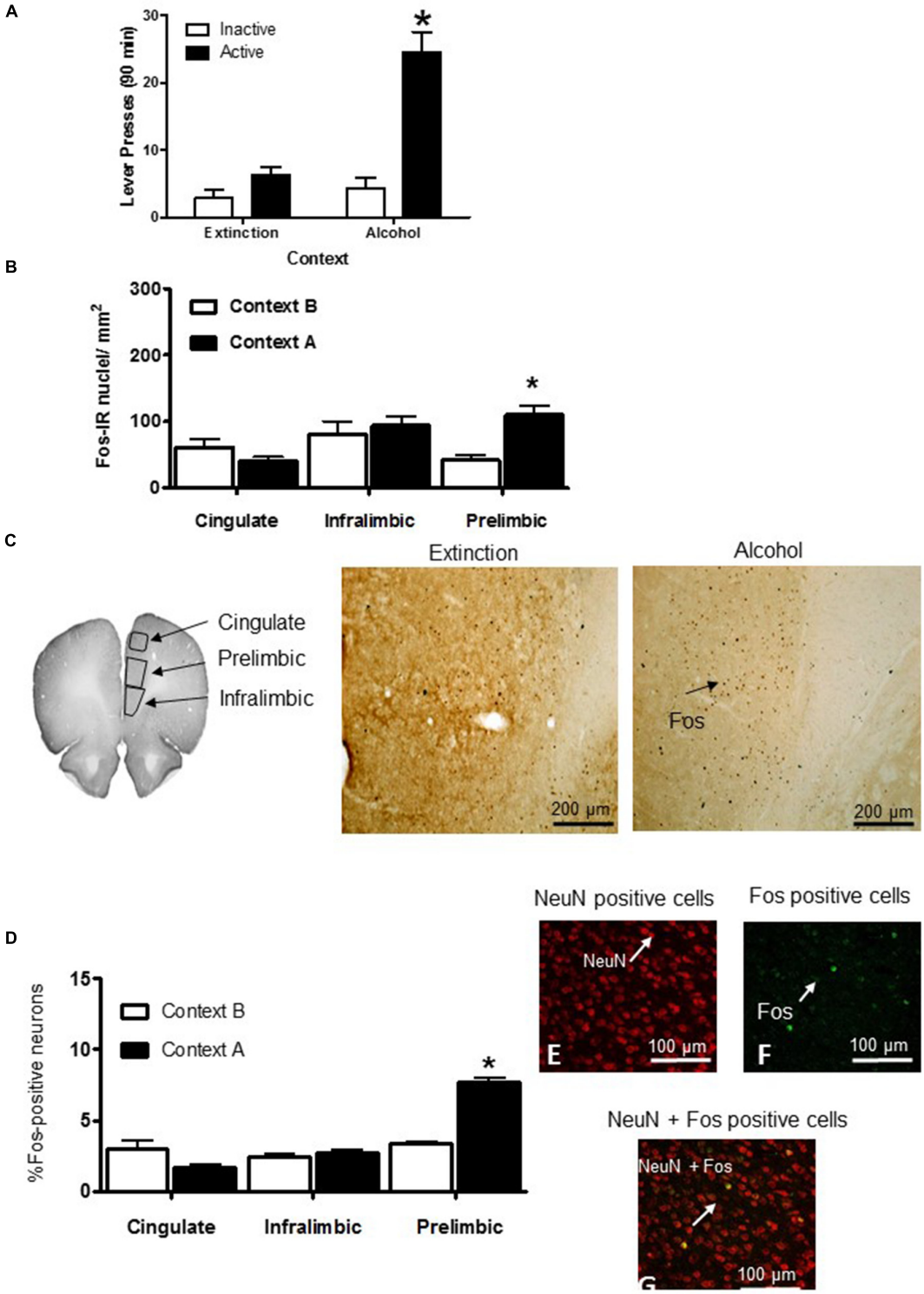

FIGURE 4 | Context-induced reinstatement of ethanol seeking increased the Fos expression in the prelimbic cortex. Data depict the mean (±standard error of the mean). **(A)** Test: Total number of active lever and inactive lever presses in rats tested in contexts A and B. *Different from context B, $p < 0.05$, $n = 9$–17 per group. **(B)** Number of Fos-immunoreactive (IR) nuclei per mm^2 in the cingulate, infralimbic, and prelimbic cortex. **(C)** Areas used for quantifying the Fos-IR neurons in the cingulate, infralimbic, and prelimbic cortex of rats. *Different from context B, $p < 0.05$ ($n = 9$–17 per group). **(D)** Percentage of neural activation by double-labeling with Fos and NeuN [(Fos^+/$NeuN^+$cells) × 100] per mm^2 in the cingulate, infralimbic, and prelimbic cortex. **(E)** Red: neuronal nuclei marker NeuN. **(F)** Green: Fos expression. **(G)** Yellow: Fos + NeuN double-labeled nuclei. Scale bar = 100 µm, $n = 4$ per group.

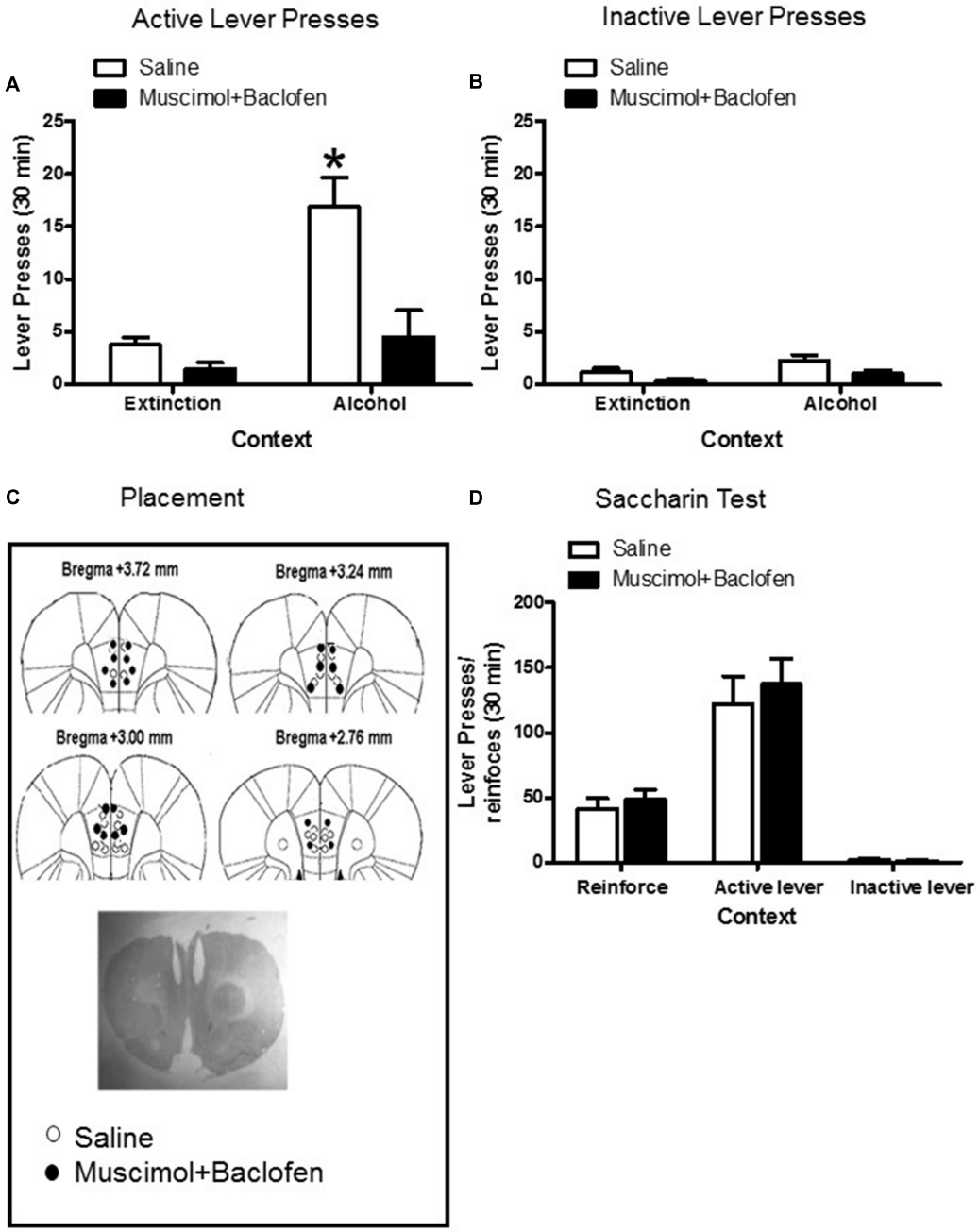

FIGURE 5 | Muscimol + Baclofen inactivation of the prelimbic cortex decreased the context-induced reinstatement of ethanol self-administration. **(A)** Total active lever-presses during the context-induced reinstatement of ethanol seeking on the test day. **(B)** Total inactive lever-presses during the context-induced reinstatement of ethanol seeking on the test day ($n = 6$–9 per group). **(C)** Dots indicate the approximate area of the injector tip. **(D)** Local inactivation of the prelimbic cortex with muscimol+baclofen had no effect on high-rate 0.2% saccharin reinforcements responding ($n = 8$–10 per group). Data are depicted as mean ± standard error of the mean. *Different from extinction context B, $p < 0.05$.

Finally, to rule out the possibility that this effect was due to motor deficits, we re-trained 18 rats that previously participated in Experiment 4 to lever presses for a 0.2% saccharin solution. After a stable responding was observed, we determined the effect of muscimol + baclofen or vehicle injections into the prelimbic cortex on high-rate operant responding for saccharin. The local inactivation of the prelimbic cortex with muscimol + baclofen had no effect on high-rate saccharin responding, indicating that the observed muscimol + baclofen effects on the context-induced reinstatement of ethanol seeking were not due to motor deficits (Interaction [lever presses/reinforcements *versus* saline/baclofen]: $F_{1,47} = 0.44$; $p = 0.51$; **Figure 5D**).

DISCUSSION

We investigated the role of the prelimbic cortex in the context-induced reinstatement of ethanol seeking. In line with previous reports (Fuchs et al., 2005; Crombag et al., 2008), ethanol seeking was induced by re-exposure to the context A after the extinction of drug self-administration in a different context. The context-induced ethanol seeking increased the expression of the neural activity marker Fos in 7.7% of the prelimbic neurons *versus* 2.5% and 1.1% in the infralimbic and cingulate cortex, respectively. The reversible inactivation of the prelimbic cortex using the GABA agonists muscimol + baclofen attenuated the context-induced reinstatement of ethanol seeking.

Context-induced reinstatement of extinguished drug seeking has been observed with several drugs of abuse including speedball (a heroin-cocaine combination) (Crombag and Shaham, 2002), cocaine (Cruz et al., 2014), heroin (Bossert et al., 2012), nicotine (Diergaarde et al., 2008), and alcoholic beer (Willcocks and McNally, 2013). Our results also corroborated previous clinical studies indicating that a context previously associated with ethanol use often provokes relapse during abstinence (O'Brien et al., 1990; Kirk and de Wit, 2000; Litt and Cooney, 2000).

Our results also corroborate with earlier studies showing that the alcohol associated context induced reinstatement of alcohol self-administration (Marchant et al., 2013; Willcocks and McNally, 2013). However, in the present study, we used a different protocol. For instance, Willcocks and McNally (2013) used an alcoholic beer, while we used an ethanol diluted in water. Further, Marchant et al. (2013) used punishment (footshock) for inducing suppression of ethanol seeking and Cannella et al. (2016) used ethanol preferring instead of Long-Evans rats for showing the ability of context induces reinstatement of ethanol seeking. The use of an alcoholic beer requires an additional control group (non-alcoholic beer). Footshock-induced extinction of ethanol seeking is not a useful procedure for studying plasticity related to context-induced relapse, because footshock might cause its own plasticity. Thus, our procedure may be considered an easy, reliable, robust, and alternative model to explore the mechanisms of context-induced relapse of ethanol seeking.

Distinct brain regions from the medial cortex have been implicated in the extinction and reinstatement of drug seeking (Fuchs et al., 2005; Lasseter et al., 2010; Bossert et al., 2011; Willcocks and McNally, 2013; Marchant et al., 2015). Our findings demonstrated a critical role of the prelimbic cortex in the context-induced relapse to ethanol seeking, which is consistent with previous studies (Willcocks and McNally, 2013). Willcocks and McNally (2013) showed that the reversible inactivation of the dorsal medial cortex by muscimol + baclofen attenuated the context-induced reinstatement of alcoholic beer seeking. There is a body of evidence indicating a role of the prelimbic cortex in different forms of reinstatement of drug seeking (Lasseter et al., 2010; Shen et al., 2014; Marchant et al., 2015; Stefanik et al., 2016). For instance, McFarland and Kalivas (2001) demonstrated that the pharmacological inactivation of the prelimbic cortex attenuated the primed reinstatement of cocaine seeking. Furthermore, Fuchs et al. (2005) demonstrated that the inactivation of the prelimbic cortex prevented the cue-induced reinstatement of cocaine self-administration. Taken together, these studies suggested that the prelimbic cortex could be a common pathway for relapse.

Evidence has also implicated the prelimbic cortex in associative learning and retrieval of remote long-term memory (Quinn et al., 2008; Euston et al., 2012). In particular, studies have demonstrated that the prelimbic cortex receives information from the emotion-related structure that is important for learning and associative memory (Peters et al., 2009; Euston et al., 2012). Furthermore, the prelimbic cortex receives a broad range of sensory and limbic inputs from the hippocampus, amygdala, orbital frontal cortex, and ventral tegmental area, which can be activated by contextual cues (Miller, 2000a,b; Mulder et al., 2000; Miller and Cohen, 2001). Additionally, the activation of the medial cortex–nucleus accumbens core–ventral pallidum pathway has been implicated in context-induced reinstatement of drug seeking (Kalivas, 2008; Peters et al., 2008; Van den Oever et al., 2010). Furthermore, it was demonstrated that the activation of these inputs can lead to context-dependent outcomes (Miller and Cohen, 2001).

Moreover, the glutamatergic and GABAergic neurons in the prelimbic cortex receive stimuli-specific patterns of inputs from the cortex, amygdala, and ventral tegmental area, which play a critical role in cognitive and emotional regulation and memory consolidation (Brandstatter et al., 1995). For instance, the hippocampal projections landing cells in the prelimbic cortex provide an essential input by which the spatial information can be integrated into the cognitive process (Floresco et al., 1997). Based on these previous findings, we assume that the activation of the prelimbic cortex by contextual cues could increase drug-seeking behaviors and cause relapse to drug use (Marchant et al., 2015).

Learned associations are hypothesized to be encoded within sparsely distributed neuronal patterns, called neuronal ensembles (Pennartz et al., 1994, 2004; Guzowski et al., 2004; Buzsaki and Moser, 2013; Cruz et al., 2013, 2015). We found that the context-induced reinstatement of ethanol seeking correlated with Fos induction in approximately 7.7% of the neurons in the prelimbic cortex. Previous studies have demonstrated the involvement of the neuronal ensembles in context-induced reinstatement of drug seeking (Cruz et al., 2013, 2014, 2015; Leão et al., 2015; Rubio et al., 2015). In some of these studies, the drug-associated contexts increased the Fos expression in the cortex and nucleus accumbens, while the selective inactivation of these Fos-expressing neurons attenuated the drug seeking behavior when exposed again to the drug-associated contexts and cues on the test day (Bossert et al., 2011; Cruz et al., 2014). These data indicated that the ability of contexts to induce reinstatement of drug seeking is mediated by specific patterns of Fos-expressing neuronal ensembles that are selected by these contexts (Bossert et al., 2011; Cruz et al., 2013, 2014, 2015; Rubio et al., 2015; Warren et al., 2016). Thus, our study suggested that a small neuronal subset of the prelimbic cortex encodes the learned association between ethanol and the drug-associated context, and that the reactivation of this small neuronal subset may lead to the reinstatement of ethanol seeking behavior. However, more

studies are necessary to demonstrate a causal role of the prelimbic neuronal ensembles in the context-induced reinstatement of ethanol seeking.

CONCLUSION

We described a new rat model of context-induced relapse to ethanol and confirmed morphologically and functionally the role of the prelimbic cortex in the context-induced reinstatement of ethanol seeking. Additionally, we demonstrated that the context-induced reinstatement of ethanol seeking was correlated with the activation of a small subset of neurons in the prelimbic cortex.

AUTHOR CONTRIBUTIONS

PP, RL, PB, PdO, CP, and FC designed the behavioral and histochemistry experiments. PP, RL, PB, PdO, and FC performed the behavioral experiments, while PP, RL, PB, and FC performed the histochemistry experiments. RL, FC, PP, RL, PB, PdO, CP, and FC wrote the paper.

ACKNOWLEDGMENT

This research was supported by São Paulo Research Foundation FAPESP (2013/24896-2).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 17 November 2017
doi: 10.3389/fphar.2017.00842

Differential Effects of Inactivation of Discrete Regions of Medial Prefrontal Cortex on Memory Consolidation of Moderate and Intense Inhibitory Avoidance Training

*María E. Torres-García, Andrea C. Medina, Gina L. Quirarte and Roberto A. Prado-Alcalá**

Departamento de Neurobiología Conductual y Cognitiva, Instituto de Neurobiología, Universidad Nacional Autónoma de México, Querétaro, Mexico

It has been found that the medial prefrontal cortex (mPFC) is involved in memory encoding of aversive events, such as inhibitory avoidance (IA) training. Dissociable roles have been described for different mPFC subregions regarding various memory processes, wherein the anterior cingulate cortex (ACC), prelimbic cortex (PL), and infralimbic cortex (IL) are involved in acquisition, retrieval, and extinction of aversive events, respectively. On the other hand, it has been demonstrated that intense training impedes the effects on memory of treatments that typically interfere with memory consolidation. The aim of this work was to determine if there are differential effects on memory induced by reversible inactivation of neural activity of ACC, PL, or IL produced by tetrodotoxin (TTX) in rats trained in IA using moderate (1.0 mA) and intense (3.0 mA) foot-shocks. We found that inactivation of ACC has no effects on memory consolidation, regardless of intensity of training. PL inactivation impairs memory consolidation in the 1.0 mA group, while no effect on consolidation was produced in the 3.0 mA group. In the case of IL, a remarkable amnestic effect in LTM was observed in both training conditions. However, state-dependency can explain the amnestic effect of TTX found in the 3.0 mA IL group. In order to circumvent this effect, TTX was injected into IL immediately after training (thus avoiding state-dependency). The behavioral results are equivalent to those found after PL inactivation. Therefore, these findings provide evidence that PL and IL, but not ACC, mediate LTM of IA only in moderate training.

Keywords: medial prefrontal cortex, overtraining, memory consolidation, anterior cingulate cortex, prelimbic cortex, infralimbic cortex, inhibitory avoidance, state-dependent learning

OPEN ACCESS

Edited by:
Antonella Gasbarri, University of L'Aquila, Italy

Reviewed by:
Patrizia Campolongo, Sapienza Università di Roma, Italy
Carlos Tomaz, Universidade Ceuma, Brazil

***Correspondence:**
Roberto A. Prado-Alcalá
prado@unam.mx

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *30 September 2017*
Accepted: *06 November 2017*
Published: *17 November 2017*

Citation:
Torres-García ME, Medina AC, Quirarte GL and Prado-Alcalá RA (2017) Differential Effects of Inactivation of Discrete Regions of Medial Prefrontal Cortex on Memory Consolidation of Moderate and Intense Inhibitory Avoidance Training. Front. Pharmacol. 8:842. doi: 10.3389/fphar.2017.00842

INTRODUCTION

A large body of research has shown that interference with neural activity shortly after a learning experience results in a significant deficiency of memory consolidation (McGaugh, 1966, 2000; Lechner et al., 1999; Izquierdo and McGaugh, 2000), lending strong support to the consolidation hypothesis put forward by Müller and Pilzecker (1900). This hypothesis implies that memory fixation requires time (consolidation) and that memory is vulnerable during the period of consolidation. This hypothesis, however, does not account for memory storage under some conditions of learning.

It has been found that varying the amount of training has important consequences on memory processes. When learning is brought about through intense training, memory formation is guarded against a host of amnestic treatments (For a review see Prado-Alcalá et al., 2012).[1] This protective effect has been consistently found after training of instrumental tasks, where a reinforcer is available after performance of a specific response (Prado-Alcalá and Cobos-Zapiaín, 1977, 1979; Prado-Alcalá et al., 1980). This effect has also been described in tasks that entail both classical and instrumental components such as active and inhibitory avoidance (IA). In these cases, the animal is exposed to a conditioned stimulus and then to an unconditioned aversive stimulus, regardless of its behavior. However, after training, the animals can avoid the aversive stimulation by performing an instrumental response before the onset of the stimulus. Thus, interfering with serotonergic activity impairs both acquisition and retention of active avoidance after training with relatively low foot-shock intensities, but not when training with higher foot-shock intensities (Galindo et al., 2008). Similarly, electrolytic lesions of lateral and basal nuclei of the amygdala impaired acquisition of a Sidman avoidance task but enhanced training protected performance of this task (Lazaro-Muñoz et al., 2010).

Systemic amnestic treatments also impede memory consolidation of IA but, again, no such deficit is produced after intense training (Durán-Arévalo et al., 1990; Cruz-Morales et al., 1992; Solana-Figueroa et al., 2002; Díaz-Trujillo et al., 2009). The same is true when treatments are administered to the striatum (Giordano and Prado-Alcalá, 1986; Pérez-Ruiz and Prado-Alcalá, 1989; Salado-Castillo et al., 2011), hippocampus (Quiroz et al., 2003; Garín-Aguilar et al., 2014), amygdala (Parent et al., 1992, 1994; Thatcher and Kimble, 1966), and substantia nigra (Cobos-Zapiaín et al., 1996).

The medial prefrontal cortex (mPFC), which includes the anterior cingulate (ACC), prelimbic (PL), and infralimbic (IL) regions (Heidbreder and Groenewegen, 2003; Vertes, 2004, 2006), has received a good deal of attention in relation to its involvement in classical fear conditioning (Mello e Souza et al., 1999; Yang and Liang, 2014; Zhang et al., 2011), and it has been suggested that these regions participate differentially across the various stages of memory of fear conditioning (Giustino and Maren, 2015). Thus, ACC has been associated with acquisition (Sacchetti et al., 2003; Tang et al., 2005; Bissière et al., 2008), PL with expression (retrieval) (Blum et al., 2006; Vidal-Gonzalez et al., 2006; Corcoran and Quirk, 2007) and IL with the process of extinction (Quirk and Mueller, 2008) and control of fear (Sotres-Bayon and Quirk, 2010). However, literature on the participation of the mPFC in memory processes related to instrumental performance is scarce. It has been reported that electrolytic lesions of IL, but not of PL, produce a deficit of the instrumental component involved in retention of step-down IA (Jinks and McGregor, 1997).

[1]Intense or enhanced training refers to conditions where a high number of trials or training sessions are given, and, in the case of aversive conditioning, to training motivated by relatively high intensities of foot-shock. In both instances, intense training yields stronger resistance to extinction than moderate and low levels of training.

To the best of our knowledge, the protective effect on learning and memory of enhanced training has not been studied in relation to selective inactivation of neural activity of ACC, PL, and IL. For this reason, and because of the differential functional attributes that have been described within the mPFC, we deemed it important to explore the effects of temporary inactivation of the three regions of mPFC on memory consolidation of moderate and intense IA training. We hypothesized that transient inactivation of AC, PL, and IL would produce differential effects on memory consolidation of IA, and that intense training would offset potential deficiencies produced by such inactivation.

MATERIALS AND METHODS

This section describes the procedures common to all the experiments of this study. Other procedures characteristic of particular experiments will be described where appropriate.

Subjects

Male Wistar rats (300–350 g) from the breeding colony at the Instituto de Neurobiología, Universidad Nacional Autónoma de México, were individually housed with water and food *ad libitum* and maintained in a room with a 12 h/12 h light-dark cycle (lights on at 7:00 h). The temperature of the room was 23 ± 1°C. The rats were randomly assigned to each group, and training and testing were performed during the light phase of the cycle, between 8:00 am and 12:00 pm. The experimental protocol was approved by the Animal Ethics Committee of Instituto de Neurobiología, Universidad Nacional Autónoma de México and complied with the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011).

Surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), injected with atropine (1 mg/kg, ip) to prevent obstruction of the respiratory tract, and their heads were positioned on a stereotaxic frame (Stoelting Co., United States). The tips of the bilateral stainless steel guide cannula (length: 8 mm for ACC and 10 mm for PL and IL; 23-gauge) were aimed 1 mm above ACC (+2.8 mm from bregma; ±0.4 mm from midline; −1.4 mm below skull), PL (+3.0 mm from bregma; ±0.7 mm from midline; −3.2 mm below skull), or IL (+3.0 mm from bregma; ±0.6 mm from midline; −4.2 mm below skull surface) (Paxinos and Watson, 2007). The cannulae were affixed to the skull using one jewelry screw and dental cement. Stylets (8 mm-long for ACC, and 10 mm-long for PL and IL) were inserted into each cannula to maintain patency and were removed, and placed back, during the manipulation sessions and for the administration of treatments. After surgery, the animals received 1.0 ml of 0.9% saline solution, ip, and were kept in an incubator until fully recovered from anesthesia. Following surgery, rats were allowed to recover for 7 days before initiation of training. During this period, each animal was handled by the experimenter, gently touching and holding the rat for approximately 5 min on three consecutive days.

Apparatus

The rats were trained in an IA apparatus consisting of two compartments separated by a sliding door. The safe compartment (30 cm × 30 cm × 30 cm) had a lid and walls made of transparent red-colored acrylic, with a floor made of stainless steel bars (6 mm in diameter, 9 mm apart). This compartment was illuminated by a 10-W light bulb located in the center of its lid. The other, non-illuminated shock compartment (30 cm long) had front and back walls and floor made of stainless steel plates with side walls and lid constructed of transparent red-colored acrylic. The walls and floor were shaped like a trough, 20 cm wide at the top and 8 cm wide at the bottom. In the middle of the floor, a 1.5 cm slot separated the two stainless steel plates that make up the walls and floor. Upon entering the non-illuminated compartment, the rats were in contact with both plates through which a foot-shock could be delivered. A square-pulse stimulator (Grass model S-48), in series with a constant current unit (Grass model CCU-1), generated the foot-shock. Shock delivery and measurement of latencies to cross from one compartment to the other one were accomplished by use of automated equipment. Both compartments were wiped with 10% alcohol before and after each rat occupied it. The apparatus was located inside a dark, sound-proof room provided with background masking noise.

Training and Testing of Inhibitory Avoidance

On the day of training, each rat was placed inside the safe compartment, and 10 s later the door between the two compartments was opened. The latency to cross from the safe compartment to the shock compartment is referred to as the training latency. Once the animals crossed to this compartment the door was closed and foot-shock of 1.0 or 3.0 mA was delivered (a train of 50 ms square pulses at 10 Hz). Five seconds later the door was reopened, allowing the rat to escape to the safe compartment, and then the stimulator was turned-off; this latency is referred to as the escape latency. After 30 s in the safe compartment, the rat was placed back in its home cage. Retention of the task was measured 48 h after training; in some cases retention was recorded both at 30 min (during encoding acquisition) and 48 h after training in the same animals. In these retention sessions, the same procedure as in training was followed except that the foot-shock was omitted. If the rat did not cross within 600 s, the session ended and a score of 600 was assigned.

Treatments

Tetrodotoxin (TTX) was used to inactivate the target areas; it reversibly blocks voltage-dependent sodium channels, thus preventing the generation and propagation of action potentials (Fozzard and Lipkind, 2010). The simultaneous bilateral infusions of TTX (Sigma, C11H17N3O8, T8024; 0.3 μg/hemisphere, dissolved in 0.3 μL of isotonic saline) or an equal volume of the vehicle (VEH) into ACC, PL, or IL were made 25 min before training. In additional groups of rats, the same dose of TTX or VEH was administered into IL immediately after training. The infusion rate was 0.3 μL/min and was controlled by an automated microinfusion pump (WPI, model 220i). At the end of the infusion, the injection needles, which protruded 1.0 mm beyond the tip of the cannulae, remained inside the guide cannulae for 60 s to minimize backflow. The injection procedure was carried out in a different room from that in which training and testing took place.

Histology

The rats were anesthetized with sodium pentobarbital (125 mg/kg) and were perfused intracardially with 0.9% saline solution followed by 4% formalin. The brains were removed and immersed in a 4% formaldehyde solution for at least 5 days. Sections were cut (50 μm thick) on a cryostat and stained with cresyl violet. The sections were examined under a light microscope, and the location of the injection needle tips was determined. The data of rats with cannula tips outside the target areas were not included in the statistical analyses. **Figures 1C**, **2F**, and **3F** show examples of cannula tip sites in ACC, PL, and IL, respectively.

c-FOS Immunohistochemistry

To evaluate the blocking effect of TTX on neural activity in each of the three regions of mPFC that were studied, we used immunohistochemistry to detect c-Fos, as this protein is commonly used as a marker of such activity (Sagar et al., 1988; Herrera and Robertson, 1996; Willoughby et al., 1997). To this end, for each region a group of rats was trained with 1.0 or 3.0 mA, and half the group was treated with TTX or VEH, as described above, but retention of the task was not measured. A group of naïve animals ($n = 6$), used to obtain the basal number of c-Fos-positive cells, was kept under identical living conditions as those of the rest of the groups, but they never left the bioterium, except for sacrificing. One hour after training, the animals were anesthetized with sodium pentobarbital (125 mg/kg) and transcardially perfused with physiological saline followed by 4% PFA (pH 9.5, 10°C). The brains were removed and stored in the fixing solution for 4 h, then in 15% sucrose overnight followed by 30% sucrose; solutions were kept at 4°C. Three days later, four serial coronal sections (30 μm in thickness) were obtained at −20°C from ACC, PL, and IL and kept in a cryoprotectant solution (30% ethylene glycol and 20% glycerol in 0.05 M sodium phosphate buffer) at −20°C until histochemical processing. The brain slices were successively incubated in PB 0.1 M for 20 min, H_2O_2 0.03% for 10 min, $NaBH_4$ 1% for 6 min, and NGS 3% for 30 min. They were then incubated for 48 h at 4°C in a c-Fos polyclonal antibody (Anti-c-Fos rabbit, 1:5000, Abcam, Cambridge, MA, United States), followed by 1 h incubation in goat anti-rabbit biotinylated secondary antibody (BA-1000, 1:500; Vector Laboratories, Burlingame, CA, United States), 1 h in a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, United States), and 10 min in DAB solution (0.03% H_2O_2, NAS). The brain slices were placed on glass slides, dehydrated progressively with alcohol followed by the clearing agent xylene, and then covered with Entellan®.

Digital images were obtained with a Leica AF6000 Microsystem (Leica, Germany) using a 10× objective. c-Fos-positive cell count was automatically performed with the

"cell counter" plug-in using the ImageJ software (NIH)[2]. Three counting boxes (100 μm × 100 μm) were positioned horizontally and centered 100 μm below the bilateral injection needle tracks; thus, six images per animal were analyzed. Because ACC, PL, and IL are next to each other along a dorsal-ventral dimension, it was important to assess the possibility that TTX might have had an effect due to diffusion from the target region to its neighboring ventral region. To this end, a counting box was positioned at 600 μm below each injection needle-tip track of the PL group. The expression level of c-Fos in each brain region for each group was expressed as the ratio of averaged count of c-Fos-positive cells of each rat for each group divided by the average count of c-Fos-positive cells for the corresponding naïve group.

Statistical Analyses

Because the measurement of retention of the IA task was truncated at 600 s, non-parametric statistics were used in analyzing the behavioral results. Comparisons of training, escape, and retention latencies between TTX and VEH groups in each region of mPFC were carried out using the Mann–Whitney *U* test. Likewise, c-Fos-positive cell counts in TTX and VEH groups in each region of mPFC were compared with the Mann–Whitney *U* test.

RESULTS

Anterior Cingulate Cortex

Training and Escape Latencies

The Mann–Whitney *U* test showed that there were no significant differences in latency scores between the TTX and VEH groups, regardless of foot-shock intensities that were used during training. Median training latencies of the VEH and TTX groups that had been trained with 1.0 mA were 15.85 and 30.35 s ($p = 0.15$), and for those trained with 3.0 mA were 19.70 and 31.05 s ($p = 1.0$), respectively (data not shown). Similarly, there were no significant differences in escape latencies between the TTX and VEH groups, regardless of the foot-shock intensities. Median escape latencies displayed by the 1.0 mA groups were 4.10 and 2.30 s ($p = 0.57$), respectively. In the VEH and TTX groups trained with 3.0 mA, escape latencies were 1.35 and 1.90 s ($p = 0.68$), respectively (data not shown).

Long-Term Memory

No significant differences between the VEH and TTX groups were evident in retention latencies measured 48 h after training, regardless of the intensity of foot-shock used for training (1.0 mA, $p = 0.46$ and 3.0 mA, $p = 0.81$) (**Figure 1A**).

c-Fos Immunohistochemistry

Tetrodotoxin administration into ACC induced a significant reduction of c-Fos expression relative to VEH in the groups that had been trained with 1.0 and 3.0 mA ($p < 0.05$ for each intensity) (**Figure 1B**). **Figure 1C** is a representative photomicrograph showing placement of a cannula tip in ACC.

[2]http://rsb.info.nih.gov/ij/

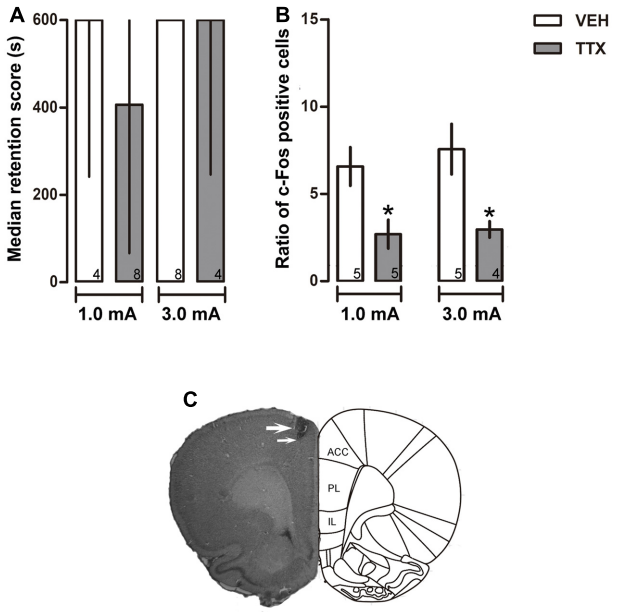

FIGURE 1 | Anterior cingulate cortex. **(A)** Median retention scores (with interquartile ranges) of groups of rats trained in one-trial IA and injected 25 min before training with the vehicle solution (VEH) or TTX into anterior cingulate cortex (ACC). TTX was ineffective in altering memory consolidation of moderate (1.0 mA) or intense (3.0 mA) training. **(B)** TTX produced a reduction of c-Fos expression close to the injector tip. $*p < 0.05$ vs. VEH. **(C)** Representative photomicrograph showing location of cannula (thick arrow) and injection needle tips (thin arrow). Numbers inside bars represent sample size.

Prelimbic Cortex

Training and Escape Latencies

The Mann–Whitney *U* test showed that there were no significant differences in training latencies displayed by the 1.0 mA TTX (40.20 s) and VEH (26.20 s) groups ($p = 0.18$), as well as in the 3.0 mA TTX (28.80 s) and VEH (26.40 s) groups ($p = 1.0$). Similarly, there were no significant differences in escape latencies between the TTX and VEH groups, regardless of the foot-shock intensities. Median escape latencies displayed by the 1.0 mA groups were 1.80 and 1.40 s, respectively ($p = 0.49$), and median escape latencies of the TTX and VEH groups trained with 3.0 mA were 0.80 and 0.60 s, respectively ($p = 0.27$) (data not shown).

Long-Term Memory

The TTX group trained with 1.0 mA displayed a significantly lower score than its VEH control group ($p < 0.05$) during the 48-h retention test. In contrast, no differences were found when comparing the TTX and VEH groups trained with 3.0 mA ($p = 0.13$) (**Figure 2A**).

Acquisition

To evaluate whether the amnestic effect of pre-training infusion of TTX in the 1.0 mA PL group was due to interference with learning of the IA task rather than with consolidation, TTX or VEH was administered 25 min before training with 1.0 mA, and

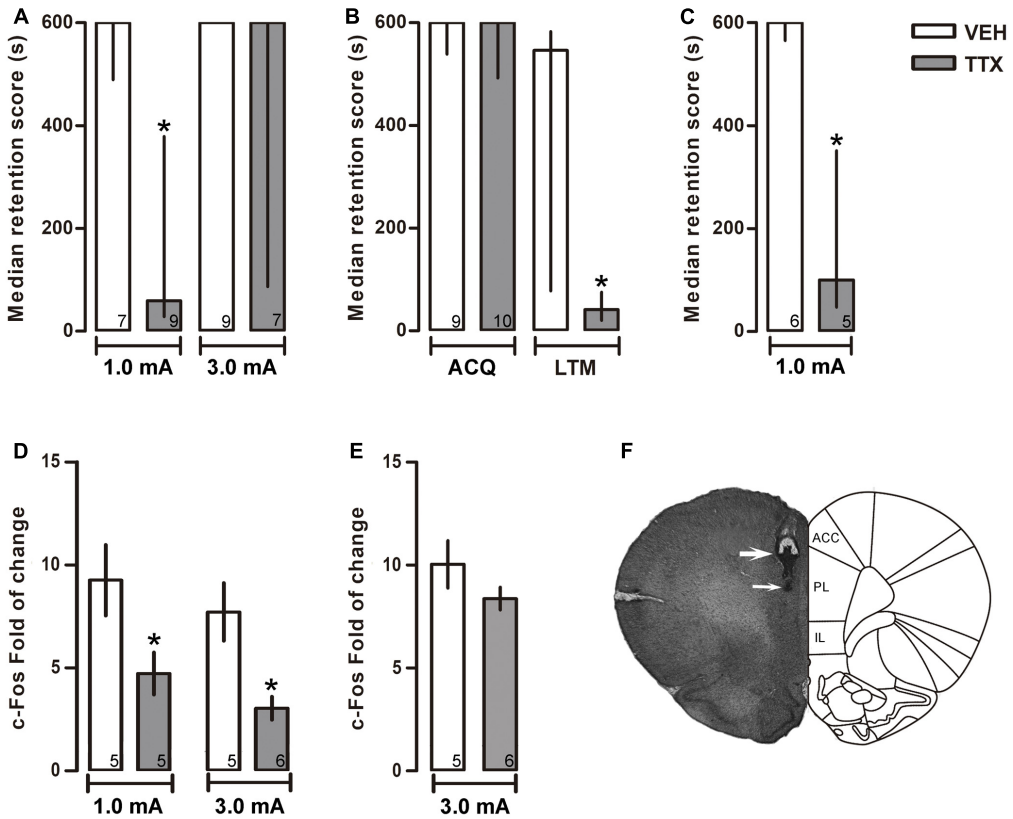

FIGURE 2 | Prelimbic cortex. Median retention scores (with interquartile ranges) of groups of rats trained in one-trial IA and injected 25 min before training with VEH or TTX into prelimbic cortex (PL). **(A)** TTX impaired memory consolidation of moderate (1.0 mA), but not of intense (3.0 mA), training. **(B)** TTX did not interfere with acquisition (ACQ) but significantly impaired long-term memory (LTM). **(C)** TTX did not produce state-dependency; TTX and VEH were administered twice, 25 min before training and 25 min before the retention test. A retention deficit was observed only after the TTX treatment. **(D)** TTX produced a reduction of c-Fos expression close to the injector tip. *$p < 0.05$ relative to VEH. **(E)** TTX did not change c-Fos expression beyond 600 μm below of cannula tip. **(F)** Representative photomicrograph showing location of cannula (thick arrow) and injection needle tips (thin arrow). Numbers inside bars represent sample size.

retention was measured twice: at 30 min and at 48 h after training. The results showed no significant differences between the TTX and VEH groups on the retention test run 30 min after training ($p = 0.95$) while, again, a reliable deficit was shown by the TTX group in the 48-h test ($p < 0.05$) (**Figure 2B**).

State Dependency

Because the TTX was administered 25 min before training, and retention was measured 48 h later in a non-drug state, it was feasible that the amnesia thus produced could have been due to a state-dependent effect. To rule out this possibility, two groups of rats were treated twice, with either TTX or VEH, 25 min before training and 25 min before retention testing. In comparison to the VEH group, the TTX group showed reliable amnesia ($p < 0.05$) (**Figure 2C**).

c-Fos Immunohistochemistry

Tetrodotoxin administration into PL induced a significant decrement in c-Fos expression relative to VEH in the groups that had been trained with 1.0 or 3.0 mA ($p < 0.05$, in each case) (**Figure 2D**).

As mentioned in Section "Materials and Methods," c-Fos expression was also measured within a 100 μm × 100 μm counting box located 600 μm below the PL injector-tip tracks. We found that TTX did not interfere with c-Fos expression, as there were no significant differences between the VEH and TTX groups, $p = 0.73$ (**Figure 2E**). This finding demonstrates that the deficit in memory consolidation seen in the animals that had been trained with 1.0 mA was due to inactivation of PL and not to diffusion of the drug into the more ventrally located IL.

Figure 2F is a representative photomicrograph showing placement of cannula tip in PL.

Infralimbic Cortex

Training and Escape Latencies

The Mann–Whitney *U* test showed that there were no significant differences in training latencies between the TTX and VEH groups, regardless of the foot-shock intensities. Median training latencies displayed by the 1.0 mA groups were 33.60 and 25.20 s ($p = 0.80$), respectively. In the VEH and TTX groups trained with 3.0 mA, training latencies were 24.20 and 23.50 s ($p = 0.97$), respectively (data not shown). Similarly, there were no significant

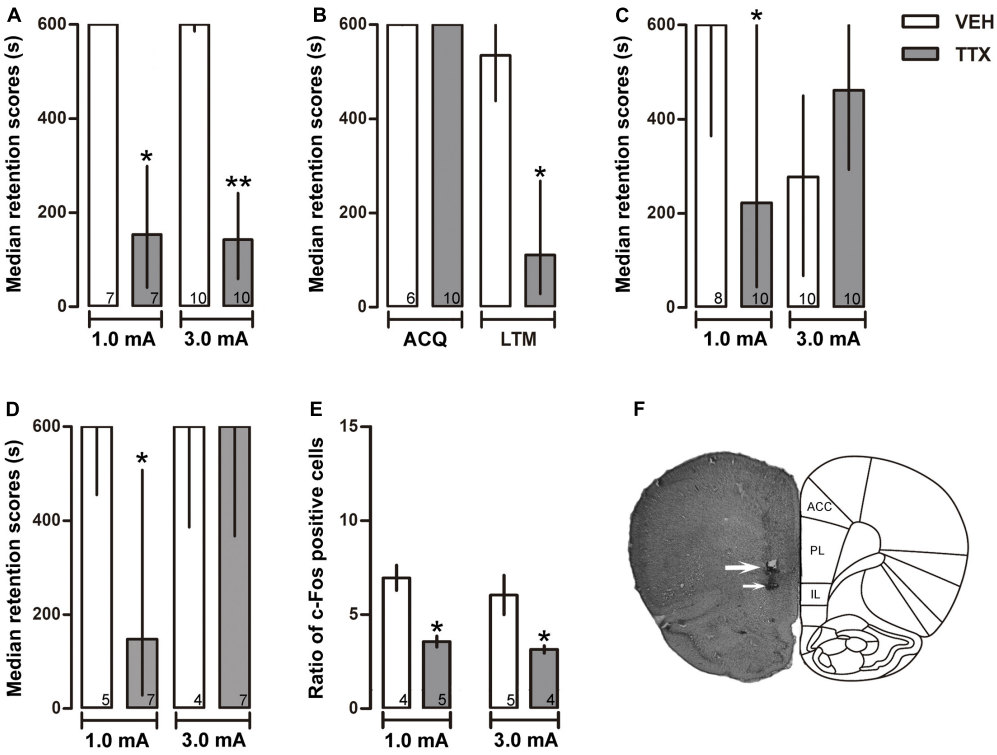

FIGURE 3 | Infralimbic cortex. Median retention scores (with interquartile ranges) of groups of rats trained in one-trial IA and injected 25 min before training with VEH or TTX into infralimbic cortex (IL). **(A)** TTX impaired retention of moderate (1.0 mA) and intense (3.0 mA) training. **(B)** TTX did not interfere with acquisition but significantly impaired LTM. **(C)** TTX and VEH were administered twice, 25 min before training and 25 min before the test of retention. A retention deficit was observed in the moderate-trained TTX group, and state-dependency was produced in the intense-trained TTX group that showed good retention of the task. **(D)** Immediate post-training administration of TTX produced a deficit in memory consolidation after moderate training (1.0 mA) but not after intense training (3.0 mA). **(E)** TTX produced a reduction of c-Fos expression close to the injector tip. $^*p < 0.05$ relative to VEH. **(F)** Representative photomicrograph showing location of cannula (thick arrow) and injection needle tips (thin arrow). Numbers inside bars represent sample size.

differences in escape latencies between the TTX and VEH groups, regardless of the foot-shock intensities. Median escape latencies displayed by the 1.0 mA groups were 1.70 and 1.20 s ($p = 0.28$), respectively. In the VEH and TTX groups trained with 3.0 mA, escape latencies were 2.70 and 2.30 s ($p = 0.65$), respectively (data not shown).

As in the case of PL, TTX infusion into IL produced a significant retention deficit during the 48-h post-training session in the group that had been trained with 1.0 mA ($p < 0.01$ vs. VEH). Unexpectedly, TTX produced the same amnestic effect in the 3.0 mA group ($p < 0.005$ vs. VEH) (**Figure 3A**).

Acquisition

To evaluate whether the amnestic effect of pre-training infusion of TTX had been due to interference with learning rather than with consolidation, two groups of rats were trained with the low foot-shock (1.0 mA) and subjected to TTX or VEH injections into the IL 25 min before training. Retention was measured twice: at 30 min and at 48 h after training. The results showed no significant differences between the TTX and VEH groups during the retention test run 30 min after training ($p = 0.85$), while a reliable deficit was shown by the TTX group in the 48-h test ($p < 0.02$) (**Figure 3B**).

State Dependency

To determine whether the amnestic effect of pre-training TTX seen in the 1.0 and 3.0 mA IL groups during the 48-h retention test (**Figure 3A**) might have been due to state-dependency, two groups of rats were trained with 1.0 mA and another two groups were trained with 3.0 mA. Half of each group was treated twice with TTX and the other half with VEH, also twice, 25 min before training and 25 min before retention testing. TTX produced a significantly lower retention score relative to its VEH control group after training with 1.0 mA group ($p < 0.05$). In contrast, state-dependency was produced when 3.0 mA was used for training, as there were no reliable differences in retention scores between the TTX and VEH groups that had been trained with 3.0 mA ($p = 0.11$) (**Figure 3C**).

Post-training TTX Administration into IL

The results of the preceding experiment showed that pre-training TTX infusion into the IL produced a clear state-dependent effect when training was conducted with 3.0 mA, but not when 1.0 mA was used. Thus, the apparent amnestic effect produced after a single pre-training TTX infusion (**Figure 3A**) could be explained by the interaction of the high foot-shock and the differential pharmacological state of the IL cortex during training (drugged

state) and retention testing (non-drugged state). This outcome did not allow us to answer the question of whether IL has a role in memory consolidation when a high aversive stimulation is used to produce learning. To shed light into this matter, we decided to study the effects of IL inactivation with TTX induced after training, thus avoiding the confounding effect of state-dependency. To this end, two groups of rats were trained with 1.0 mA and another two groups were trained with 3.0 mA. Half of each group was treated with TTX and the other half with VEH. The infusions were made immediately after training. A significant retention deficit was observed in the TTX group that had been trained with 1.0 mA ($p < 0.03$ vs. VEH), whereas no significant differences between the TTX and VEH groups trained with 3.0 mA were found ($p = 0.11$) (**Figure 3D**).

c-FOS Immunohistochemistry

The infusion of TTX into the IL induced a significant decrement in c-Fos expression relative to VEH in the groups that had been trained with 1.0 or 3.0 mA ($p < 0.05$ for each comparison) (**Figure 3E**).

DISCUSSION

The main findings of this study, where TTX was administered before training, were that regardless of the intensity of training, transient inactivation of ACC did not disrupt memory consolidation of the IA task. In contrast, in PL and IL TTX produced a highly significant deficit of consolidation when the moderate foot-shock was used in training. Interestingly, the retention deficit was still evident after training with the high foot-shock when IL had been inactivated, due to state-dependency, but retention was not diminished in the PL group (**Figures 1A**, **2A**, **3A**). When TTX was administered immediately post-training into IL, it interfered with consolidation only when the moderate foot-shock was used. We suggest that these differential effects are dependent on the dissimilar connectivity of the three regions that were studied. They receive strong connections from the same thalamic regions; PL and IL receive afferents from the basolateral and basomedial nuclei of the amygdala; and PL is more densely connected to limbic cortical areas than ACC and IL (Hoover and Vertes, 2007). Further research is needed to study the contribution of these different anatomical interactions in memory consolidation of IA.

The histochemical results showed that administration of TTX in each of those cortical regions produced reliable neuronal inactivation, as evidenced by the diminished detection of c-Fos near the injector tips. Because there were no significant differences in training and escape latencies between the TTX- and VEH-treated animals, irrespective of the microinjected region or the intensity of the foot-shock used for training, the impaired retention that was found in the PL and IL groups cannot be explained by any potential deficiency of the motor or perceptual activities necessary to perform the IA task. In other words, the treated animals could cross from the safe compartment to the shock compartment, and escape from the foot-shock just as efficiently as the VEH-treated animals. What follows is a discussion focusing on relevant studies on IA.

Anterior Cingulate Cortex

Inactivation of ACC did not interfere with memory consolidation of IA, as indicated by the high retention scores of animals trained with 1.0 and 3.0 mA. This agrees with the report of Mello e Souza et al. (1999) that intra-ACC administration of muscimol and AP5 did not impede the formation of long-term memory of this task. Our result is also congruent with the lack of impairment of memory consolidation of IA found after pre-training radiofrequency lesion (Chai et al., 2010) of ACC. Taken together, these data suggest that this region is not involved in neural activity encoding needed for memory consolidation of the CS-UCS association during training of the IA task. This interpretation must be taken cautiously, because other lines of research suggest that ACC is involved in memory consolidation of IA. Thus, infusion of the cholinergic agonist oxotremorine into ACC immediately after IA training improved memory (Malin and McGaugh, 2006) and, consistent with this finding, it was shown that pre-training and post-training infusion of scopolamine, a cholinergic antagonist, impaired memory consolidation of this task (Riekkinen et al., 1995). Furthermore, administration of a protein synthesis inhibitor into ACC or mPFC (which included PL and IL) produced a significant retention deficit of IA (Zhang et al., 2011). New studies are needed to comprehend these dissimilar results.

Prelimbic Cortex

The findings that inactivation of PL produced a marked deficiency of retention when the low intensity foot-shock was used for training, and that it did not impede performance when the high foot-shock was used (**Figure 2A**) fit well with previous results where interference with neural activity of striatum (Giordano and Prado-Alcalá, 1986; Pérez-Ruiz and Prado-Alcalá, 1989), hippocampus (Quiroz et al., 2003; Garín-Aguilar et al., 2014), amygdala (Parent et al., 1992, 1994), and substantia nigra (Cobos-Zapiaín et al., 1996; Salado-Castillo et al., 2011) disrupted memory consolidation when IA training took place with a low intensity of aversive stimulation, but not when stimulation of relatively high intensity was used.

That the impairment in retention shown by the TTX group that had been trained with the low foot-shock was due to interference with memory consolidation, and not to a deficiency in learning, was demonstrated by the optimal performance shown by the group of animals that was tested 30 min after the administration of the drug. A deficit in consolidation became evident when this group was given a second retention test 48 h later (**Figure 2B**).

Because training took place under the influence of TTX, and retention of the task was measured when the animals were in a non-drugged condition, the possibility existed that the retention deficit observed in the low foot-shock group was due to a phenomenon of state-dependency and not to disturbance of memory consolidation. This possibility was

discarded because a group of rats that was trained and tested under the same pharmacological condition exhibited a deficient retention (**Figure 2C**).

The retention deficit observed in the present study gives support to the findings of Santos-Anderson and Routtenberg (1976) and of Jinks and McGregor (1997). The former authors showed that low-level electrical stimulation of the ventral aspect of the mPFC interfered with memory consolidation of IA, and the latter found that electrolytic lesions of PL produced a deficit in IA. Together, these findings indicate that PL has similar functions to those of the striatum, hippocampus, amygdala, and substantia nigra regarding memory consolidation of IA, i.e., these structures are necessary for memory consolidation under conditions of moderate training because interference with neural activity of any one of them impedes the formation of long-term memory. On the other hand, consolidation takes place after intense training despite this interference. It has been hypothesized that these structures are not critical for mediating associative processes derived from intense training, which produces plastic changes allowing for the recruitment of other structures.

Infralimbic Cortex

The study of IL yielded a complex set of data. In agreement with previous results, where lesions of IL produced a significant retention deficit of IA (Jinks and McGregor, 1997), we found that infusion of TTX into IL had the same detrimental effect when the low foot-shock was used for training. Contrary to our expectations, intense training did not protect memory consolidation against the inactivation produced by the TTX (**Figure 3A**). The memory test that was made 30 min post-training to the group of animals that were treated with TTX into IL yielded top retention scores, while a reliable deficit was observed 48 h later in this same group. As in the case of PL inactivation, these results indicate that learning took place and that the impaired retention was due to a failure in memory consolidation (**Figure 3B**).

When TTX was infused into IL twice (both before training and before the 48-h retention test) a retention deficit was produced in the low foot-shock group but, unexpectedly, not in the high foot-shock group (**Figure 3C**). To the best of our knowledge, this is the first time that a state-dependent effect has been found in mPFC. Thus, the question of whether IL is involved in memory consolidation of intense training could not be answered with this experimental design. This problem was solved by administering the treatments immediately after training with the low and the high intensity of foot-shock, thus avoiding the induction of state-dependency. This manipulation confirmed that inactivation of IL impedes memory consolidation when IA training is carried out with an aversive stimulus of low intensity, and it revealed that, indeed, intense training protects against the amnestic effect of inactivation produced by the TTX (**Figure 3D**).

CONCLUSION

The data obtained in this experimental series indicate that (a) memory consolidation of IA is not dependent on neural activity of the ACC; (b) normal activity of PL is essential for memory consolidation of moderate IA training, but not for acquisition or for consolidation of intense training; (c) normal activity of IL is also essential for memory consolidation of moderate IA training but not for acquisition or for consolidation of intense training. Moreover, the combined effect of TTX and intense training induces state-dependency.

AUTHOR CONTRIBUTIONS

Designed the experiments: MT-G, AM, and RP-A. Performed the behavioral and histological experiments: MT-G and AM. Analyzed the data: MT-G and AM. Wrote and provided comments and discussion for the manuscript: MT-G, AM, GQ, and RP-A.

FUNDING

This research was supported by Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México (UNAM; IN201415) and by Consejo Nacional de Ciencia y Tecnología (CONACYT; 237570). MT-G is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, UNAM and received fellowship 245656 from CONACYT.

ACKNOWLEDGMENTS

The authors thank Bertha Islas, Norma Serafín, Omar González, Leonor Casanova, Martín García, and Sandra Hernández for their excellent technical and administrative assistance. We also wish to thank Jessica González Norris for editorial comments.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 12 December 2017
doi: 10.3389/fphar.2017.00849

Potential Therapeutic Effects of Lipoic Acid on Memory Deficits Related to Aging and Neurodegeneration

Patrícia Molz[1, 2] *and Nadja Schröder*[1, 2]*

[1] *Graduate Program in Medicine and Health Sciences, Faculty of Medicine, Pontifical Catholic University, Porto Alegre, Brazil,* [2] *Neurobiology and Developmental Biology Laboratory, Faculty of Biosciences, Pontifical Catholic University, Porto Alegre, Brazil*

The aging process comprises a series of organic alterations, affecting multiple systems, including the nervous system. Aging has been considered the main risk factor for the advance of neurodegenerative diseases, many of which are accompanied by cognitive impairment. Aged individuals show cognitive decline, which has been associated with oxidative stress, as well as mitochondrial, and consequently energetic failure. Lipoic acid (LA), a natural compound present in food and used as a dietary supplement, has been considered a promising agent for the treatment and/or prevention of neurodegenerative disorders. In spite of a number of preclinical studies showing beneficial effects of LA in memory functioning, and pointing to its neuroprotective potential effect, to date only a few studies have examined its effects in humans. Investigations performed in animal models of memory loss associated to aging and neurodegenerative disorders have shown that LA improves memory in a variety of behavioral paradigms. Moreover, cell and molecular mechanisms underlying LA effects have also been investigated. Accordingly, LA displays antioxidant, antiapoptotic, and anti-inflammatory properties in both *in vivo* and *in vitro* studies. In addition, it has been shown that LA reverses age-associated loss of neurotransmitters and their receptors, which can underlie its effects on cognitive functions. The present review article aimed at summarizing and discussing the main studies investigating the effects of LA on cognition as well as its cell and molecular effects, in order to improve the understanding of the therapeutic potential of LA on memory loss during aging and in patients suffering from neurodegenerative disorders, supporting the development of clinical trials with LA.

Keywords: memory, lipoic acid, neuroprotection, aging, neurodegenerative disorders

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***Correspondence:**
Nadja Schröder
nadja.schroder@pucrs.br

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *26 June 2017*
Accepted: *06 November 2017*
Published: *12 December 2017*

Citation:
Molz P and Schröder N (2017) Potential Therapeutic Effects of Lipoic Acid on Memory Deficits Related to Aging and Neurodegeneration. Front. Pharmacol. 8:849. doi: 10.3389/fphar.2017.00849

INTRODUCTION

Aging is a multifactorial process that involves genetics, lifestyle, and environmental factors (Hagen et al., 1999; Savitha and Panneerselvam, 2006; Lopez-Otin et al., 2013; Kennedy et al., 2014). During aging, biological processes promote the gradual loss of the individual's ability to maintain homeostasis, followed by a progressive deterioration in biochemical and physiological functions of the organism, increasing the susceptibility to diseases associated with aging (Arivazhagan et al., 2001a; Kumaran et al., 2005; Singh et al., 2015). Cognitive function also declines with age (Liu, 2008).

Aging has been associated to a more oxidized state in the redox balance (Jones and Sies, 2015), and the central nervous system becomes vulnerable to oxidative stress (Arivazhagan et al., 2002; Kidd, 2005; Ferreira et al., 2013; Zuo and Motherwell, 2013), defined as an unbalanced redox signaling, related to increased amounts of oxidants and ineffective antioxidant defenses (Go and Jones, 2017). In this context, nutrition can be considered a critical life-style factor that impacts the development and progression of neurodegenerative diseases (Virmani et al., 2013). Dietary supplementation with mitochondrial nutrients could promote natural neuroprotective effects, delaying the onset or progression of cognitive dysfunction and neurodegenerative diseases (Miquel, 2002; Abadi et al., 2013; Di Domenico et al., 2015; Mehrotra et al., 2015).

Over the years, lipoic acid (LA) has received increased attention as a nutritional supplement with therapeutic potential in the treatment or prevention of different pathologies (Shay et al., 2009; Rochette et al., 2013; Park et al., 2014), such as neurodegenerative diseases (Packer et al., 1997; Hager et al., 2001; Holmquist et al., 2007; Farr et al., 2012). LA has been shown to improve mitochondrial function (Kidd, 2005; Zhang et al., 2010; Zuo and Motherwell, 2013; Hiller et al., 2016), in addition to protect from cognitive dysfunction associated to aging and neurodegenerative diseases (Hager et al., 2007; Moreira et al., 2007; Liu, 2008). Thus, this study aims to review and discuss main findings showing potential memory-improving effects, as well as neuroprotective effects of LA, giving support to its use as an adjuvant in the treatment of neurodegenerative disorders. For this, we focused in discussing experimental studies addressing behavioral evaluations and cellular and molecular effects of LA.

LIPOIC ACID

LA (1,2-dithiolane-3-pentonoico acid, thioctic acid) (Shay et al., 2009) was discovered in 1937 by Snell et al. (1937) and characterized by Reed et al. (1951). LA has been considered a powerful micronutrient presenting a range of pharmacological properties (Rochette et al., 2013; Koufaki, 2014; Park et al., 2014); however, many aspects of LA effects still need to be clarified, especially its application in the treatment and prevention of neurodegeneration.

Chemistry of LA

LA is a low molecular weight dithiol with a chiral center containing eight-carbon disulfide in its structure (**Figure 1**; De Araujo et al., 2011). It is naturally occurring in all prokaryotic and eukaryotic cells (Bast and Haenen, 2003; Park et al., 2014). LA acts as co-factor in multienzyme complexes in the mitochondria, such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Holmquist et al., 2007; Ghibu et al., 2009). A substantial part of LA is reduced to dihydrolipoic acid (DHLA) by lipoamide dehydrogenase (E3 component of the pyruvate dehydrogenase complex and α-ketoglutarate dehydrogenase) with involvement of the NADH and NADPH system (Arivazhagan et al., 2001b; Bilska et al., 2007). Reduction of LA to DHLA may also be completed by other cellular reducing systems, including NAD(P)H-driven enzymes, such as thioredoxin reductases (Rochette et al., 2013). LA also contains an asymmetric carbon resulting in two optical isomers, the S form and the R form, with the former being synthesized endogenously (De Araujo et al., 2011).

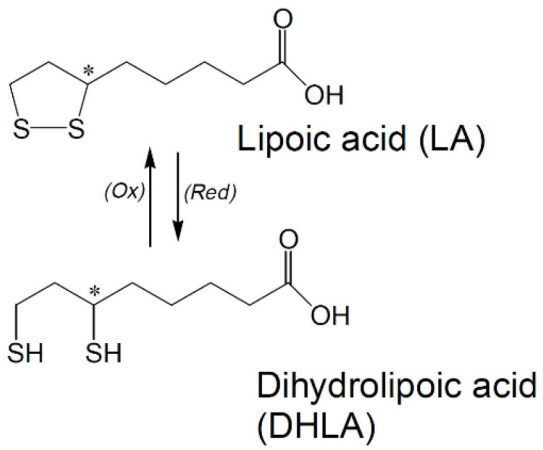

FIGURE 1 | Chemical structure of lipoic acid (LA) and dihydrolipoic acid (DHLA). *Indicates a chiral Carbon atom.

Dietary Sources, Metabolism, Toxicity, and Nutritional Recommendations

LA may be obtained from the diet (Packer et al., 1997; Hagen et al., 1999; Morikawa et al., 2001; Ghibu et al., 2009) and due to its capability of endogenous synthesis it is not considered a vitamin (Packer et al., 1995, 2001; Bast and Haenen, 2003), but is structurally considered a member of the vitamin B family (Xing et al., 2015). When ingested as a nutritional supplement preferably in the racemic mixture form, LA contains two isomers (1:1 of R-LA and S-LA), in which S-LA may prevent polymerization of R-LA and thus increases the bioavailability of the later (De Araujo et al., 2011).

LA is found in both vegetable and animal-based foods, identified as lipoyl-lysine (coupling of LA to specific lysine residues; Ghibu et al., 2009; Shay et al., 2009; Rochette et al., 2013; Li et al., 2015). R-LA is most abundantly found in vegetables such as spinach, broccolis, and tomatoes, which contain, respectively, 3.15 ± 1.11, 0.94 ± 0.25, and $0.56 \pm 0.23 \times 10^{-3}$g lipoyl-lysine (gram dry weight). In foods of animal origin, the highest amounts of lipoyl-lysine are found in bovine kidney, heart, and liver, containing, respectively, 2.64 ± 1.23, 1.51 ± 0.75, and $0.86 \pm 0.33 \times 10^{-3}$ g lipoyl-lysine (gram dry weight) (Lodge and Packer, 1999).

The biochemistry and pharmacokinetics of LA have been extensively reviewed elsewhere (Packer et al., 1995; Bustamante et al., 1998; Cremer et al., 2006b; Shay et al., 2009; Rochette et al., 2013). In summary, either from food or nutritional supplement sources, LA is rapidly absorbed, metabolized and excreted (Moini et al., 2002; Rochette et al., 2013). LA is absorbed rapidly from the gastrointestinal tract (Hagen et al., 1999; Jalali-Nadoushan

and Roghani, 2013), up to 93% of a dose administered orally is absorbed in the gastrointestinal tract, (Cremer et al., 2006b) and is clearly subject to considerable pre-systemic elimination (Hagen et al., 1999). Approximately 27–34% LA orally administered is available for absorption by the tissues (Hagen et al., 1999) and the liver is one of the main clearance organs because it has a high absorption and storage capacity (Bustamante et al., 1998; Hagen et al., 1999).

Studies have shown that the gastrointestinal absorption of LA is highly variable and its efficiency appears to be reduced when ingested in the diet, suggesting that the absorption of LA competes with other nutrients (Packer et al., 2001; Shay et al., 2009; Rochette et al., 2013). Gastrointestinal uptake of LA is fast and its presence in the plasma is followed by a fast clearance (Shay et al., 2009). The plasmatic half-life of LA is 30 min. Endogenous plasma levels of LA and DHLA in humans are, respectively, 1–25 $\times 10^{-9}$ g/mL and 30–140 $\times 10^{-9}$ g/mL (Ghibu et al., 2009; Rochette et al., 2013). Urinary excretion is maximal 3–6 h following LA administration. Approximately 45% is excreted in the urine during the first 24 h and only 3% is excreted in the feces. Moreover, only a small amount of the administered LA is excreted in the unaltered form (Bustamante et al., 1998).

There are no recommendations for daily LA intake in humans. However, the safety of LA was determined in acute and sub-chronic toxicity studies as well as its mutagenicity/genotoxicity in *in vitro* and *in vivo* studies. In dogs, a LD_{50} of 400–500 mg LA/kg b.w. has been reported (Packer et al., 1995). In rats a LD_{50} of 2,000 mg/kg b.w. was described, with some rats presenting signs of reduced well-being, including sedation, apathy, piloerection, hunched posture, and/or eye closure (Cremer et al., 2006b). On the other hand, studies evaluating oral LA supplementation up to 60 mg/kg per day in rats showed no adverse effects concerning body weight, histopathological findings, and blood analyses (Cremer et al., 2006a,b). Therefore a NOAEL (no observed adverse effect level) for rats of 60 mg/kg/day for long-term LA supplementation was provided. Clinical trials using LA to assess adverse health effects in humans were performed in doses up to 2,400 mg/day with no reported adverse effects vs. placebo (Shay et al., 2009). In spite of that, the exact doses that could induce adverse human health effect are still to be set up.

NEURODEGENERATIVE DISORDERS

Neurodegenerative diseases are a heterogeneous group of disorders described by progressive and selective neuronal death with degeneration of specific brain regions (Arivazhagan and Panneerselvam, 2002; Lin and Beal, 2006; Savitha and Panneerselvam, 2006), often associated with abnormal deposits of proteins in neurons or extracellularly (Chen et al., 2012). Neurodegeneration is characterized by its insidious and chronic progressive onset and aging has been considered the main risk factor (Lin and Beal, 2006; Chen et al., 2012; Virmani et al., 2013; Irwin et al., 2016).

During aging, deleterious changes accumulate, causing the gradual decline of the biochemical and physiological functions (Arivazhagan and Panneerselvam, 2002; Kumaran et al., 2005; Santos et al., 2013). Moreover, aging individuals are susceptible to degeneration of selective brain regions (Aliev et al., 2009), increasing the incidence of diseases such as Alzheimer's, Parkinson's, Huntington's diseases, among others (Lin and Beal, 2006; Bagh et al., 2011; Irwin et al., 2016).

Over the last decades, a wide range of studies have shown that progression of neurodegeneration is associated with increased DNA damage (partly is attributed to an imbalance between antioxidant and prooxidant factors; Chen et al., 2012; Kim et al., 2015). In addition, mitochondrial decline, leading to cognitive dysfunction (Liu, 2008; Aliev et al., 2009; Bishop et al., 2010; Irwin et al., 2016) as well as aggregation of oxidized proteins, accumulation of metals, inflammation and excitotoxicity have been reported (Moreira et al., 2007). Oxidative stress and mitochondrial dysfunction are interrelated mechanisms that play a central role in aging brain (Santos et al., 2013), since the continuous generation of reactive oxygen species (ROS), mainly the superoxide anion ($O_2^{-\bullet}$) at complexes I and III of the mitochondrial respiratory chain, throughout life produces a sustained oxidative stress by aging, possibly resulting in cognitive impairments (Limoli et al., 2004; Kumaran et al., 2005; Bagh et al., 2011). Although the exact mechanisms underlying the effects of unbalanced redox signaling are not completely elucidated, studies suggest that it plays a role in the pathogenesis of neurodegenerative disorders (Liu et al., 2017).

Brain aging has been related to structural alterations and inflammation, accompanied by cognitive and memory dysfunctions (Bagh et al., 2011; Pizza et al., 2011; Thakurta et al., 2014). With the aging of the world population and the increasing life expectancy, the risk of developing neurodegenerative diseases is higher than ever, as they are affecting millions of people each year in epidemic proportions (Santos et al., 2013; Irwin et al., 2016). As a result, an inevitable socioeconomic burden on our health care systems will occur. Effective prophylactic and therapeutic treatments are urgent for this group of seemingly inexorable diseases. In this review we will focus on discussing the ameliorating effects of LA on cognitive deficits observed in animal models of aging and neurodegenerative disorders.

EFFECTS OF LA IN EXPERIMENTAL MODELS OF MEMORY DEFICITS ASSOCIATED TO NEURODEGENERATIVE DISORDERS

Behavioral Studies

Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder that causes dementia and affects middle to old-aged individuals (Hager et al., 2001; Gonzalez et al., 2014; Irwin et al., 2016). AD is characterized by progressive loss of cognitive functions, including memory, language, and reasoning (Di Domenico et al., 2015).

Studies have investigated the effects of LA in experimental AD models (Jesudason et al., 2005; Siedlak et al., 2009; Ahmed, 2012;

Sancheti et al., 2013). For instance, Quinn et al. (2007) evaluated the chronic dietary supplementation with LA on hippocampus-dependent memory of aged Tg2576 mice, a transgenic model of cerebral amyloidosis associated with AD. LA treatment was shown to reduce hippocampal-dependent memory deficits, significantly improving learning and memory in the Morris water maze in comparison to Tg2576 mice that did not receive LA. However, no significant differences in β-amyloid levels were found between Tg2576 mice that received LA in comparison to the ones that did not receive LA, indicating that chronic LA supplementation in the diet can ameliorate hippocampal memory impairments in Tg2576 mice without any effect on β-amyloid levels or plaque deposition.

Another study assessed the effects of LA in senescence-accelerated mouse prone 8 (SAMP8) mice, associated to learning and memory impairments, and showed that LA can improve memory, in different paradigms (Farr et al., 2012). In object recognition, results indicated that mice that received LA presented a higher memory index than vehicle-treated mice. When memory was tested in the Barnes maze, results indicated that LA-treated mice spent more time by the target, and made fewer errors than controls, but did not present differences in speed in traversing the maze (distance/time) during training.

On the other hand, Siedlak et al. (2009) investigated young and aged mice overexpressing amyloid-β precursor protein (APP) and controls and showed that administration of R-LA had little effect on Y-maze performance. The authors concluded that, although oxidative stress has been proposed to mediate amyloid pathology and cognitive decline in aging, long-term LA administered within tolerable nutritional levels, presented limited benefit.

Parkinson's Disease

Parkinson's disease (PD) is the second most frequent neurodegenerative disorder in aging individuals and features motor symptoms related to dopaminergic neuronal loss in the *substantia nigra*, which results in decreased striatal dopaminergic terminals (Beal, 2003; De Araujo et al., 2011). Studies have shown that, in addition to rescuing cognitive deficits, LA is also able to ameliorate motor impairment related to PD. The effects of LA were examined in a rat model of PD induced by rotenone. The effect of LA (50 mg/kg/day, p.o.) was evaluated after the administration of rotenone in the open-field and square bridge tests. The authors reported that LA improved rotenone-induced behavioral deficits. In the open-field test, LA significantly increased the ambulation frequency, increased the number of stops, elevated the activity index and lessened the inactive sittings, but did not increase the rearing frequency in comparison to the group that received rotenone. In the Square bridge test, treatment with LA protected the rats from falling as compared to rotenone group (Zaitone et al., 2012).

Jalali-Nadoushan and Roghani (2013) investigated the effect of LA (at doses of 50 and 100 mg/kg) in a 6-hydroxydopamine (6-OHDA)-induced model of hemi-parkinsonism and observed significantly attenuated rotations on behavioral testing, induced by both doses.

The effects of LA in lipolysaccharide (LPS)-induced inflammatory PD model were also evaluated (Li et al., 2015), and the results showed that LA treatment (100 mg/kg/d) partially improved motor dysfunction. No significant recovery was observed in dyskinesia in PD mice that received LA. However, a significant amelioration was observed in the adhesive removal test, in which LA treatment significantly decreased the reaction time in comparison to the LPS group.

Huntington's Disease

Huntington's disease (HD) is a chronic neurodegenerative disease and a hereditary autosomal-dominant disorder of the central nervous system caused by a single genetic mutation (Ross et al., 2014), characterized by neuronal death in caudate and putamen and in the cerebral cortex, and to a lesser extent in hippocampus and subthalamic nucleus (Mehrotra et al., 2015). This disorder is classically characterized by motor symptoms and cognitive and behavioral features (Ross et al., 2014). A HD model that has been easily replicated in animals is based on the treatment with 3-nitropropionic acid (3-NP), which promotes development of mitochondrial dysfunctions leading to bioenergetic failure (energy impairment, oxidative stress, and excitotoxicity). A study by Mehrotra et al. (2015) investigated the effects of LA in 3-NP-induced HD in rats. Administration of LA improved spatial memory acquisition and retrieval assessed using the Morris water maze. Analysis of time taken and distance traveled to find the platform in the target quadrant revealed that LA supplementation for 21 days to 3-NP–treated animals resulted in a lower latency and the distance traveled was also reduced. In addition, the average number of platform crossings in the probe trial was increased in 3-NP treated animals that received LA. Thus, the authors demonstrated that LA supplementation improved spatial memory by ameliorating the iron- and copper-induced oxidative injury observed in age-related disorders. In the Y-maze test, animals display a preference to explore the novel arm of the maze, making fewer entrances in the previously explored arm, due to spontaneous alternation. The authors showed that 3-NP–treated animals traveled a significantly lower distance, assessed by the number of entries in the novel arm, and that supplementation with LA reversed these deficits.

Aging Models

Consistent evidence indicate that memory is affected by aging in rodents as well as in humans. A study by Liu et al. (2002a) investigated the effects of LA supplementation (0.1% in the diet) on spatial memory tested in the Morris water maze, and temporal memory using the peak procedure (time-discrimination procedure) in old rats. Results showed that LA supplementation alone or combined with another mitochondrial metabolite, acetyl-l-carnitine, improved both spatial and temporal memory. In 12-month old SAMP-8 mice, chronic LA administration improved cognition in both the T-maze footshock avoidance paradigm and the lever press appetitive task without inducing non-specific locomotor effects (Farr et al., 2003).

LA has also been reported to improve behavior of aged mice in an open-field memory test (Stoll et al., 1993, 1994), in a Morris

water maze test (Stoll et al., 1994; Liu et al., 2002a). LA also and ameliorated acquisition and retrieval in a dose-dependent manner, in old female NMRI mice, in the active avoidance learning test (Stoll et al., 1994).

Other Models of Neurotoxicity

Cui et al. (2006) evaluating a concomitant treatment with LA and d-Galactose exposure (used to induce memory loss and neurodegeneration) verified that LA ameliorated memory dysfunction in the Morris water maze task. Another study reported the neuroprotective effects of LA in neurotoxicity model induced by $AlCl_3$ administration to mice (Mahboob et al., 2016). LA enhanced fear memory and social novelty preference in comparison to the $AlCl_3$-treated group.

In summary, current evidence indicates that LA is able to improve memory, reversing impairments associated to a variety of experimental models of neurodegenerative disorders, and exposure to neurotoxicants, as well as normal aging. **Table 1** summarizes *in vivo* studies investigating the neuroprotective effects of LA on behavioral parameters. Furthermore, LA was also shown to act as a memory-improving molecule in different learning and memory paradigms, including aversive, spatial, and recognition memory.

LA has been tested in humans, in studies by Hager and coworkers (Hager et al., 2001, 2007), as a treatment option for AD. The authors examined the effect of LA for 24 and 48 months and observed that the treatment lead to a stabilization of cognitive function, verified by unchangeable records in two neuropsychological tests, mini-mental state examination (MMSE) and the AD assessment score, cognitive subscale (ADAScog).

PUTATIVE MECHANISMS UNDERLYING LA-INDUCED NEUROPROTECTIVE EFFECTS

In vivo as well as *in vitro* studies have been performed in order to characterize cellular and molecular effects of LA underlying its memory-ameliorating activities (**Table 2**). The effects of LA on oxidative markers in various brain regions have been discussed in different studies in animals models of aging and neurodegenerative diseases (Cui et al., 2006; Ferreira et al., 2009; Militao et al., 2010; Farr et al., 2012). LA administration decreases lipid peroxidation evaluated by MDA (Arivazhagan and Panneerselvam, 2000; Arivazhagan et al., 2002; Liu et al., 2002b; Ferreira et al., 2009; Militao et al., 2010; Farr et al., 2012) in different brain regions, and elevates the activities of antioxidants such as ascorbate (vitamin C), α-tocoferol (vitamin E) (Arivazhagan and Panneerselvam, 2000), glutathione (GSH) (Arivazhagan and Panneerselvam, 2000; Farr et al., 2012), superoxide dismutase (SOD) activity (Arivazhagan et al., 2002; Cui et al., 2006; Militao et al., 2010), catalase (CAT) (Arivazhagan et al., 2002; Militao et al., 2010), glutathione peroxidase (GSH-Px) (Arivazhagan et al., 2002; Militao et al., 2010), glutathione redutase (GR) (Arivazhagan et al., 2002), glucose-6-P-dehydrogenase (G6PDH) (Arivazhagan et al., 2002). Moreover, administration of LA reversed the augmentation of protein carbonyls levels in a radiation-induced cognitive dysfunction model (Manda et al., 2007), and decreased the protein carbonyls levels in aged SAMP8 mice (Farr et al., 2003).

A study by Zaitone et al. (2012) showed that LA increased striatal dopamine levels and significantly increased GSH and CAT activity in the striatum in a PD experimental model. In reserpine-treated rats, LA enhanced the amount of GSH, while diminishing GSSG levels in the striatum. Moreover, LA decreased NO concentrations in striatum and pre-frontal cortex, without significantly affecting S-nitrosothiol levels. LA also increased enzymatic activities of GPx and GST in the striatum (Bilska et al., 2007). Reserpine significantly decreased enzymatic activity of L-γ-glutamyl transpeptidase (γ-GT), while pretreatment with LA was able to restore it.

The effects of LA on oxidative stress in rotenone parkinsonian rat brains were investigated, showing that LA can reduce lipid peroxidation and protein carbonylation (Zaitone et al., 2012). LA also lowered the levels of MDA and nitrite in the 6-OHDA-induced rat model of hemi-parkinsonism (Jalali-Nadoushan and Roghani, 2013). Karunakaran et al. (2007) analyzed the protective effect of LA in the MPTP mouse model of PD, demonstrating that coadministration with LA prevents the activation of apoptosis signal regulating kinase (ASK1) signaling cascade and translocation of Daxx (death associated protein) in ventral midbrain and striatum, attenuating dopaminergic cell loss. R-LA induced significant reductions in markers of oxidative modifications in transgenic AD mice model, significantly decreasing HO-1 and protein-bound HNE levels (Siedlak et al., 2009). Inman et al. (2013) analyzed the effect of LA in the DBA/2J mouse model of glaucoma. The results showed that after 4 and 11 months of dietary LA, respectively, LA treatment increased antioxidant genes and protein expression, protected retinal ganglion cell (RGC), and improved retrograde transport. Dietary therapy also reduced lipid peroxidation, protein nitrosylation, and DNA oxidation in a retina model of glaucoma.

Accumulation of metal ions also has been associated with increased oxidative stress related with aging and neurodegenerative disorders. Suh et al. (2005) showed that LA supplementation can modulate age-related cortical iron accumulation, acting as metal chelator, thereby ameliorating age-associated oxidative stress. However, Liu et al. (2002b) showed that high concentrations of iron and copper found in old rats were not significantly decreased with LA supplementation.

There are multiple cell death mechanisms implicated in neurodegeneration. Apoptosis is a highly controlled cellular process that can be activated by two pathways: extrinsic, which is a receptor-mediated pathway, and intrinsic, which is mediated by signals from the mitochondria. Both pathways culminate at cleavage-dependent activation of aspartate-specific effector caspases (caspases-3, 6, and 7). Cui et al. (2006), using chronic systemic exposure of d-galactose in an aging model observed that a treatment with LA decreased caspase-3 protein levels and neuronal apoptosis, ameliorating neurodegeneration in the hippocampus. Manda et al. (2007), demonstrated that LA pretreatment protected against radiation. They observed that

TABLE 1 | Summary of studies testing the effects of LA on behavioral parameters in animal models.

Experimental model	Regimen of treatment	Effects	References
Aging	Chronic, α-LA (100 mg/kg body weight, 15 days, orally	Improved spatial memory in aged animals	(Stoll et al., 1993, 1994)
Aging	Chronic, fed an AIN-93M diet with R-LA (0.5% w/w) for 2 wk.	Improved ambulatory activity in old and young animals.	(Hagen et al., 1999)
Aging	R-α-LA [0.2% or 0.1% (wt/wt) in diet] for 7 weeks	Improved spatial and temporal memory in old animals	(Liu et al., 2002a)
SAMP8 accelerated aging mouse	Chronic, LA (100 mg/kg), subcutaneously for 4 weeks	Improved memory in the T-maze footshock avoidance paradigm and the lever press appetitive task in old animals	(Farr et al., 2003)
Aging model	Chronic, α-LA (100 mg/kg body weight) daily, 7 weeks, intraperitoneally	Improved spatial memory	(Cui et al., 2006)
Radiation-induced cognitive dysfunction	Sub-chronic, LA (200 mg/kg bw) intraperitoneally for 5 days	Improved spatial memory	(Manda et al., 2007)
Model of cerebral amyloidosis	Chronic, fed an α-LA-containing diet (0.1%) for 6 months	Improved learning and memory retention; exhibited more context freezing	(Quinn et al., 2007)
Model of amyloid-β protein precursor-over-expressing	Chronic, LA (diet 30 mg/kg per day) for 10 months	No effects on spatial memory	(Siedlak et al., 2009)
Model of AD using SAMP8 mice	Chronic, LA (100 mg/kg), subcutaneously for 4 weeks	Improved spatial memory in old animals	(Farr et al., 2012)
Model of rotenone-induced parkinsonism	Sub-chronic, α-LA (50 mg/kg/day/12 doses[12 days], po)	Improved spatial memory	(Zaitone et al., 2012)
6-OHDA model of hemi-parkinsonism in rats	50 and/or 100 mg/kg 24 h before of surgery	Attenuated rotational behavior	(Jalali-Nadoushan and Roghani, 2013)
Model of LPS-induced Parkinson's disease	Sub-chronic, LA (100 mg/kg/d) administered ip for 30 days	Improved motor dysfunction	(Li et al., 2015)
Model 3-NP–induced HD	Chronic, α-LA (50 mg/kg), intraperitoneally for 21 days	Improved motor dysfunction	(Mehrotra et al., 2015)
$AlCl_3$-induced neurotoxicity	Sub-chronic, α-LA mixed in diet (200 ppm = dose of 25 mg/kg/day) for 12 days	Enhanced fear memory and social novelty preference	(Mahboob et al., 2016)

3-NP, 3-nitropropionic acid; AD, Alzheimer's disease; AlCl3, Aluminum chloride; HD, Huntington's disease; LPS, lipolysaccharide; 6-OHDA, 6-hydroxydopamine.

pre-treatment with LA prevented radiation-induced decreases of total, nonprotein and protein-bound sulfhydryl (T-SH, NP-SH, and PB-SH) levels in the cerebellum. Moreover, LA treatment also improved the cytoarchitecture of cerebellum, increasing the number of intact Purkinje cells and granular cells when compared to untreated irradiated mice.

Mehrotra et al. (2015) evaluated the effects of LA on mitochondrial dysfunctions in the 3-NP induced model of HD. The results showed that LA decreased malondialdehyde, protein carbonyls, reactive oxygen species and nitrite levels, and increased Mn-superoxide dismutase and CAT activity. They also found that LA improved histological and biochemical alterations, such as decreased cytosolic cytochrome c levels, caspase-3 and−9 activity and expression of apoptotic proteins (AIF, Bim, Bad, and Bax), suggesting its therapeutic efficacy in HD. LA improved activity of enzymes from the mitochondrial respiratory chain, altered cytochrome levels, increased histochemical staining of complex-II and IV, increased in-gel activity of complex-I to V, and increased mRNA expression of respiratory chain complexes.

Stoll et al. (1993) investigated the effect of LA on NMDA Receptor deficits in old female NMRI mice. The results showed that LA improved age-related NMDA receptor deficits (B_{max}). No changes were observed regarding muscarinic, benzodiazepine, and α_2-adrenergic receptor deficiencies. Thus, the authors concluded that LA-induced memory improving effects may be related to partial reparation of NMDA receptor deficits that accompany aging.

A loss of dopaminergic neurons is particularly relevant to PD, in which genetic and environmental factors are involved (Di Domenico et al., 2015; Li et al., 2015). Jalali-Nadoushana and Roghania using a rat model of hemi-parkinsonism (6-OHDA) found that LA prevented neuronal loss on the left side of the *substantia nigra pars compacta* (SNpc) (Jalali-Nadoushan and Roghani, 2013). In a study using the LPS-induced inflammatory PD model, Li et al. (2015) demonstrated that LA administration protected against dopaminergic neuron loss. Zaitone et al. (2012) observed that LA induced an increase in the number of neurons in the SNpc in rotenone parkinsonian rats. Li et al. (2015) reported that in addition to protecting against dopaminergic

TABLE 2 | Summary of *in vivo* studies testing cellular and molecular effects of LA.

Experimental model	Regimen of treatment	Effects	References
Aging	Chronic, α-LA (100 mg/kg body weight, orally for 15 days	LA alleviated age-related NMDA receptor deficits (B_{max})	Stoll et al., 1993
Aging	Acute and Chronic, DL-α-LA (100 mg/kg body weight/day), 7 or 14 days, young and aged rats, intraperitoneally	↓ lipid peroxidation, ↑ levels of antioxidants in various brain regions	Arivazhagan and Panneerselvam, 2000
Aging	α-LA (300 mg/kg/day) for 6weeks	↓ mtDNA deletions associated with aging	Seidman et al., 2000
Aging	Acute and Chronic, DL-α-LA (100 mg/kg body weight/day), 7 or 14 days, young and aged rats, intraperitoneally	↑levels of neurotransmitters (dopamine, serotonin and norepinephrine) in various brain regions	Arivazhagan and Panneerselvam, 2002
Aging	Acute and Chronic, DL-α-LA (100 mg/kg body weight/day), 7 or 14 days, young and aged rats, intraperitoneally	↓ level of lipid peroxidation, ↑activities of antioxidant enzymes in various brain regions	Arivazhagan et al., 2002
Aging	R-α-LA [0.2% or 0.1% (wt/wt) in diet] for 7 weeks	↓ reduced the extent of oxidized RNA, reversed age-associated mitochondrial structural decay	Liu et al., 2002a
Aging	R-α-LA [0.2% (wt/wt) in diet] for 7 weeks	Inhibited lipid peroxidation but did not decrease iron and copper levels	Liu et al., 2002b
SAMP8 accelerated aging mice	Chronic, LA (100 mg/kg), subcutaneously for 4 weeks	↓Protein carbonyl levels, ↑W/S ratio, ↓TBARS levels	Farr et al., 2003
Abeta peptide vaccination-induced inflammation	Acute, LA (100 mg kg −1 body weight), intraperitoneally for 4 days	↑ levels of 5-HT, DA and NE and the concentration of 5-HIAA and HVA gradually returned to normal	Jesudason et al., 2005
SAMP8 accelerated aging mice	Chronic, LA as a racemic mixture (100 mg/kg body weight) daily, 4 weeks, subcutaneously	↑ brain proteins (neurofilament triplet L protein, α-enolase, and ubiquitous mitochondrial creatine kinase), ↓ specific carbonyl levels of the three brain proteins (lactate dehydrogenase B, dihydropyrimidinase-like protein 2, and α-enolase)	Poon et al., 2005
Aging	Chronic, LA (0.2% [w/w]) for 2 weeks	↓cerebral iron levels, antioxidant status and thiol redox state improved markedly	Suh et al., 2005
Adult male C57BL/6 mice with D-gal administration	Chronic, α-LA (100 mg/kg body weight) daily, 7 weeks, intraperitoneally	Ameliorated neurodegeneration in the hippocampus, ↓ peripheral oxidative damage, ↑ T-AOC and T-SOD, no effect on GSH-Px, ↓ caspase-mediated apoptosis, ↑ neurogenesis and neuron migration, ↓oxidative	Cui et al., 2006
Reserpine rat model of PD	Acute, LA (50 mg/kg) administered twice, 30 min before and after reserpine injection, intraperitoneally	↑ concentration of GSH and ↓GSSG level in the striatum, ↑ GSH level and no changes in GSSG content in the prefrontal cortex, ↓ NO concentrations, ↑ Enzymatic activities of GPx and GST in the striatum	Bilska et al., 2007
MPTP model of PD	Acute, α-LA (22 mg/kg body weight, sc) twice daily, concomitant with MPTP	Abolished the activation of ASK1 and phosphorylation of downstream kinases, MKK4, and JNK and prevented the down-regulation of DJ-1 and translocation of Daxx to the cytosol; attenuated dopaminergic cell loss in SNpc	Karunakaran et al., 2007
Cognitive impairment induced by radiation	Sub-chronic, LA (200 mg/kg bw) intraperitoneally for 5 days	Protected against augmentation of protein carbonyls and TBARS in cerebellum; intact cytoarchitecture of cerebellum, higher counts of intact Purkinje cells and granular cells; T-SH, NP-SH, PB-SH contents of cerebellum and plasma FRAP was inhibited	Manda et al., 2007
Aging	Chronic, α-LA (100 mg/kg body weight/day dissolved in alkaline saline) for 30 days	Old rats: ↓ levels of mitochondrial LPO, 8-oxo-dG and oxidized glutathione and enhanced reduced glutathione, ATP, lipoic acid and ETC complex activities; Young rats: ↓ levels of LPO, 8-oxo-dG and oxidized glutathione and ↑levels of reduced glutathione, ATP, lipoic acid, TCA cycle enzymes and ETC complex activities.	Palaniappan and Dai, 2007
Tg2576 AD mouse model	Chronic, fed an α-LA-containing (0.1%) for 6 months	No effects on β-amyloid levels or plaque deposition	Quinn et al., 2007

(Continued)

TABLE 2 | Continued

Experimental model	Regimen of treatment	Effects	References
Aging	Acute, LA (10, 20 or 30 mg/kg, i.p.), 24 h	↓ lipid peroxidation level, no alteration was observed in SOD activity, ↑ DA and NE, ↓ 5-HT and their metabolites 5-HIAA, the metabolites (DOPAC and HVA) did not differ in hippocampus	Ferreira et al., 2009
Tg2576 AD mouse model	Chronic, LA (diet 30 mg/kg per day) for 10 months	↓expression of HO-1 and protein-bound HNE, ↓ HO-1 around amyloid plaques, ↓ protein-bound HNE expression surrounding Aβ plaques, Redox active iron accumulation was specifically co-localized with Aβ plaques in the hippocampus and cortical regions	Siedlak et al., 2009
Pilocarpine-induced seizures	Acute, LA (20 mg/kg, ip) for 30 min	↓ lipid peroxidation and nitrite concentrations and ↑SOD, CAT and GPx activities in striatum	Militao et al., 2010
$AlCl_3$ rat model of AD	Chronic, α-LA (100 mg/kg/day for 3 months) after $AlCl_3$ (100 mg/kg b.wt/day for 4 months), orally.	↓ AChE activity, ↓ inflammation, ↑ neuronal and regeneration features	Ahmed, 2012
SAMP8 accelerated aging mice	Chronic, LA (100 mg/kg), subcutaneously for 4 weeks	↑GSH, ↓MDA, ↓GPx	Farr et al., 2012
Rotenone rat model of PD	Sub-chronic, α-LA (50 mg/kg/day/12 doses[12 days], po)	↑striatal dopamine level, no effect on striatal ATP level, ↓ level of lipid peroxides and ↓protein carbonyls in rat brains, ↑tissue GSH, improved injury to mtDNA and normalization of the mtDNA content, ↑ percentage of SNpc dopaminergic neurons, ↑ number of Nissl stained neurons	Zaitone et al., 2012
DBA/2J mouse model of glaucoma	60 mg/kg body weight (bw)/day for the intervention study and 100 mg/kg bw/day for the prevention study in diet for 24 months	↑antioxidant gene and protein expression, ↑protection of RGCs and improved retrograde transport, ↓ lipid peroxidation, ↓ protein nitrosylation, ↓ DNA oxidation in the prevention and intervention paradigms.	Inman et al., 2013
Unilateral intrastriatal 6-OHDA-lesioned rats	50 and/or 100 mg/kg 24 h before surgery	Prevented loss of SNC neurons, ↓ levels of MDA and nitrite	Jalali-Nadoushan and Roghani, 2013
3 × Tg-AD	Chronic, LA (0.23% w/v in drinking water) for 4 weeks	↑brain glucose uptake; ↑ in the total GLUT3 and GLUT4 in the old mice; activation of the insulin receptor substrate and of the PI3K/Akt signaling pathway; changes in synaptic function (↑I/O) and LTP.	Sancheti et al., 2013
Arsenic-dichlorvos exposed rats	Chronic, α-LA (50 mg/kg/day for 10 months), orally.	Oxidative stress and cholinergic dysfunction was protected	Dwivedi et al., 2014
Lipolysaccharide (LPS)-induced inflammatory PD model	Sub-chronic, LA (100 mg/kg/d) administered ip for 30 days	Protected dopaminergic neurons loss, ↓α-synuclein accumulation in the substantia nigra, inhibited the activation of NF-κB and expression of pro-inflammatory molecules in M1 microglia	Li et al., 2015
3-NP-induced HD model of	Chronic, α-LA (50 mg/kg), intraperitoneally for 21 days	Restored respiratory chain enzyme activities, CAT activity was improved, normalized of mitochondrial appearance, stimulated the repair of mitochondrial membranes and restored functionality to impaired mitochondria, ↓lipid peroxidation, ↓protein carbonyls, ↓ROS and nitrite levels, ↓cytosolic levels of cytochrome c, ↓activities of caspase-3 and 9.	Mehrotra et al., 2015
$AlCl_3$-induced neurotoxicity mouse model	Sub-chronic, α-LA mixed in diet (200 ppm = dose of 25 mg/kg/day) for 12 days	Improves the expression of muscarinic receptors (M1 and M2) and choline acetyltransferase	Mahboob et al., 2016

3-NP, 3-nitropropionic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; 5-HT, serotonina; 6-OHDA, 6-hydroxydopamine; 8-oxo-dG, monoclonal anti-8-hydroxyguanine; AChE, acetylcholinesterase; AD, Alzheimer's disease; $AlCl_3$, Aluminum chloride: ASK1, apoptosis signal regulating kinase 1; Aβ, amyloid-β fibrils; CAT, catalase activity; DA, dopamine; D-gal, D-galactose; DOPAC, 3,4-hydroxyphenylacetic acid; ETC, electron transport chain; FRAP, ferric reducing power; GLUT3, glucose transporter 3; GLUT4, glucose transporter 4; GPx, glutathione peroxidase; GSH, reduced glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione disulfide; GST, glutathione-S-transferase; HNE, 4-hydroxynonenal; HO-1, heme oxygenase-1; HVA, homovanillic acid; I/O, input/output; JNK, Jun N-terminal kinase; LPO, lipid peroxidation; LPS, Lipolysaccharide; LTP, long term potentiation; M1,Type 1 macrophages/microglia; M2, Type 2 macrophages/microglia; MDA, malondialdehyde; MKK4, mitogen-activated protein kinase kinase 4; MPTP, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine; mtDNA, mitochondrial DNA; NE, norepinephrine; NF-κB, nuclear factor-κB; NMDA receptor, N-methyl-D-aspartate receptor; NO, nitric oxide; PD, Parkinson's disease; RGC, retinal ganglion cell; ROS, reactive oxygen species; SNC, substantia nigra pars compacta; SNpc, substantia nigra pars compacta; SOD, total superoxide dismutase; T-AOC, total antioxidative capabilities; TBARS, thiobarbituric acid reactive substance; T-SOD, total superoxide dismutase; W/S, weakly immobilized/strongly immobilized.

neuron loss, LA also decreased α-synuclein deposits in the substantia nigra (SN). Moreover, the authors showed that LA inhibited the stimulation of nuclear factor-κB (NF-κB) and expression of pro-inflammatory molecules in M1 microglia. Zaitone et al. (2012) also investigated the effect of LA on mitochondrial DNA (mtDNA) integrity and quantity in the rotenone model of PD. The results showed that LA significantly decreased rotenone-induced mtDNA damage.

Liu et al. (2002a), examined the effects of LA on mitochondrial structure, and neurodegeneration in the hippocampus, and oxidative damage to nucleic acids in the hippocampus and cortex of aged rats. Dietary administration of LA significantly reduced oxidized RNA levels and reversed mitochondrial structural deterioration induced by aging in the hippocampus. Dwivedi et al. (2014) investigated the protective efficacy of LA against co-exposure to arsenic-dichlorvos in rats. The results indicated that arsenic and dichlorvos induced oxidative stress and cholinergic dysfunction in brain, which was significantly protected by the supplementation with LA.

Seidman et al. (2000) indicated that age-associated mitochondrial impairment may be hampered by LA administration. Their results showed that mtDNA deletions associated with aging were reduced by LA and this effect appeared to be related to the mitochondrial capacity to protect and repair mtDNA against age-induced injury. Palaniappan and Dai (2007) investigated the effect of LA administration to aged rats and verified a reduction of mitochondrial lipid peroxidation, 8-oxo-dG and oxidized glutathione (GSSG) and increased GSH, ATP, and electron transport chain (ETC) complex activities in the brain.

The SAMP8 mouse strain is an experimental model that displays increased oxidative stress accompanied by memory decline associated to a rapid aging process. Proteomic analyses were used to examine differential protein expression and/or protein oxidative changes in brain samples from aged SAMP8 mice. In order to determine the mechanisms underlying LA-induced reversion of memory deficits exhibited by SAMP8 mice, Poon et al. (2005) analyzed the expression and specific carbonylation of proteins in brains from 12-month-old SAMP8 mice that received LA or vehicle. The levels of three proteins (neurofilament triplet L protein, a-enolase, and ubiquitous mitochondrial creatine kinase) were significantly increased, while protein carbonylation was reduced in lactate dehydrogenase B, dihydropyrimidinase-like protein 2, and a-enolase in aged SAMP8 mice that received LA, suggesting that, in addition to improving learning and memory, LA also can restore specific proteins in aged SAMP8 mouse brain.

Evidence indicates that deregulation in neurotransmitter systems, including decreased levels of neurotransmitters, decline in the number of receptors, and lower responsiveness to neurotransmitters can be key features of neurological disorders (Payton et al., 2005; Fidalgo et al., 2013). Arivazhagan and Panneerselvam (2002) investigated the effect of LA on levels of neurotransmitters (dopamine, serotonin, and norepinephrine), and showed that LA treatment can improve neurotransmitter function in models of neurodegenerative diseases. Jesudason et al. (2005) investigated the effect LA on levels of neurotransmitters in a model of AD by Aβ amyloid vaccination. The results showed that AD mice treated with LA exhibited enhanced levels of serotonin, dopamine, and norepinephrine, and the concentration of metabolites 5-hydroxyindole acetic acid (5-HIAA) and homovanillic acid (HVA) gradually returned to normal.

Ahmed (2012) explored the effect of LA on brain acetylcholinesterase (AChE) activity. The authors demonstrated that LA can ameliorate neurological injury related to Aβ and Al excess, by significantly restoring AChE activity. In addition, the authors showed that the treatment with LA restored the parameters of total homocysteine (tHcy), insulin, insulin like growth factor-1 (IGF-1), interlukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). Mahboob et al. (2016), analyzed the effects of LA in $AlCl_3$- model of neurodegeneration, demonstrating its capacity in ameliorating cognitive functions and enhancing cholinergic system functions. LA treatment increased the expression of muscarinic receptor genes M1, M2 and choline acetyltransferase (ChaT) relative to $AlCl_3$-treated group.

There are many studies examining the neuroprotective actions of LA using *in vitro* models of neurodegeneration (Tirosh et al., 1999; Li et al., 2013; Xing et al., 2015), most of which focus on AD. For example, Ono et al. (2006) investigated the effects of LA and its reduced form, DHLA, on the formation, extension, and destabilization of β-amyloid fibrils (fAβ). The results showed that both LA and DHLA inhibited fAβ formation from amyloid β, as well as their expansion, and undermined preformed fAβs in a dose dependent manner. Lovell et al. (2003) also studied the effects of LA and DHLA in neuronal cultures challenged with amyloid β-peptide (Aβ 25-35), and observed that DHLA, but not LA, significantly protected against neurotoxicity induced by amyloid β-peptide and iron/hydrogen peroxide (Fe/H_2O_2).

In β-amyloid-intoxicated C6 glioma cells, LA increased cell viability and MnSOD expression. The increased GSSH and decreased GSH mitochondrial levels induced by Aβ were reversed by treatment with LA (Xing et al., 2015). In addition, LA protected cortical neurons against Aβ peptide- and hydrogen peroxide-induced damage, suggesting that the neuroprotective effects were partly related to PKB/Akt signaling pathway stimulation (Zhang et al., 2001).

The study by Deuther-Conrad et al. (2001) showed that the advanced glycation end products (AGE)-induced increases in oxidized glutathione were inhibited by R-LA in SH-SY5Y human neuroblastoma cells, indicating that AGE-mediated depletion of reduced glutathione follows the production of superoxide and hydrogen peroxide. de Arriba et al. (2003) investigated the effect of R-LA, in the same types of cells, on AGE accumulation, and found that AGE-induced metabolic changes were diminished by R-LA. Tirosh et al. (1999) showed that LA protected HT4 neuronal cells against glutamate-induced cytotoxicity, by inhibiting intracellular GSH depletion, and canceled the buildup of intracellular peroxide levels following the glutamate exposure.

Kamarudin et al. (2014) showed that R-LA ameliorated glutathione over glutathione disulfide ratio, decreased intracellular ROS levels and increased mitochondrial membrane potential in NG108-15 cells. In addition, R-LA stimulated the production of an anti-inflammatory cytokine, IL-10, inactivating glycogen synthase kinase-3b (GSK-3β) and decreasing both

Bax/Bcl2 and Bax/Bcl-xL ratios. Suppression of NF-κβ p65 translocation and production of proinflammatory cytokines (IL-6 and TNF-α) followed inhibition of cleaved caspase-3. Yamada et al. (2011) investigated the effects of different isomers of LAs (racemate, R-LA, and S-LA) in human neuroblastoma SH-SY5Y cells. They showed that all types of LAs were effective in preventing cell death. R-LA and S-LA also enhanced expression of genes related to anti-oxidative response such as heme oxygenase-1 (HO-1) and phase II detoxification enzymes such as NAD(P)H:Quinone Oxidoreductase 1 (NQO1).

Other studies evaluated the effect of LA on *in vitro* model of PD. Li et al. (2013) showed that pretreatment with LA significantly prevented against apoptosis of PC12 cells elicited by MPP+, and inhibited intercellular ROS levels and mitochondrial transmembrane permeability, thereby protecting dopaminergic neuronal cells against oxidative damage. Moreover, Zhang et al. (2010) demonstrated that R-LA hindered rotenone-induced mitochondrial dysfunction, oxidative damage, and α-synuclein and ubiquitin deposition in SK-N-MC human neuroblastoma cells.

Involvement of LA in the Regulation of Cellular Signaling Pathways

LA has been proposed to exert a modulatory control on the cellular redox status. Due to its ability to be interconverted in one of its two forms—i.e., thiol, the reduced form and disulfide, the oxidized form—LA can regulate cellular redox environment by interacting with redox couples such as glutathione/glutathione disulfide, cysteine/cystine, and thioredoxin (Packer and Cadenas, 2011). LA has been described to regenerate other antioxidants, such as vitamin C and E, to increase GSH levels, and to provide modulation of proteins and transcription factors (Packer et al., 1995). Extracellular redox state is also regulated by LA, once its reduced form, DHLA, can interact with cystine, reducing it to cysteine, thereby stimulating its uptake by the cell, which in turn stimulates GSH synthesis (Han et al., 1997). Owing to the role played by LA in the regulation of thiol/disulfide redox couples, LA can be viewed as regulator of cell signaling and gene expression.

PI3K/Akt signaling pathway, critical to the regulation of cell growth, proliferation, differentiation, survival, and metabolism, has been shown to be modulated by LA. For instance, Jiang et al. (2013) have demonstrated that age-associated imbalance of PI3K/Akt was restored by LA treatment for 3 weeks in the drinking water in rats. LA treatment significantly increased Akt phosphorylation and lead to a recovery in the ratio pJNK/pAkt in cortex of aged rats. Prevention of sevoflurane-induced apoptosis by LA was accomplished through recovery of Akt and GSK3-β phosphorylation levels in the hippocampus (Ma et al., 2016). By activating PI3K/Akt signaling pathway, LA, administered for 3 days, was able to ameliorate cerebral ischemia and reperfusion-induced damage in adult rats (Dong et al., 2015). Sancheti et al. (2013) showed that LA increases brain glucose uptake and activates the insulin receptor substrate and the PI3K/Akt signaling pathway in a triple transgenic mouse model of AD (3xTg-AD), reversing the impaired synaptic plasticity and increasing input/output (I/O) and long-term potentiation (LTP).

CONCLUDING REMARKS

Compelling evidence indicates that LA displays memory-ameliorating properties in a variety of experimental models of neurodegenerative diseases, as well as in memory decline associated with aging in rodents. Studies aiming to assess the neuroprotective effects of LA on behavioral outcomes showed that LA can reduce memory deficits in different behavioral paradigms on AD (Quinn et al., 2007; Farr et al., 2012), HD (Mehrotra et al., 2015), oxidative stress (Stoll et al., 1993, 1994; Liu et al., 2002a; Farr et al., 2003; Manda et al., 2007), and age-associated cognitive dysfunction (Cui et al., 2006; Mahboob et al., 2016) models. In humans, two studies in AD patients have supported the positive cognitive effects of LA (Hager et al., 2001, 2007).

Many studies reported beneficial effects of LA in the rat brain or neuronal cell cultures, using different molecular markers of oxidative stress, such as reduction in the levels of lipid peroxides and protein carbonyls, recycling endogenous antioxidants such as vitamin C and E, increasing glutathione levels (Packer et al., 1997; Di Domenico et al., 2015; Mehrotra et al., 2015), inhibiting free radical formation, chelating transition metal ions such as iron, thus reducing its bioaccumulation in the brain (Moini et al., 2002; Shay et al., 2009; Rochette et al., 2013). LA was also shown to display anti-inflammatory properties (Deuther-Conrad et al., 2001; Li et al., 2015), and affect cell death. *In vivo* and *in vitro* studies showed that LA ameliorated neurodegeneration in the hippocampus, decreasing neuronal apoptosis and caspase-3 protein levels, supporting a neuroprotective role mediated by the mitochondrial cell death pathway. These effects suggest that LA is able to improve mitochondrial dysfunctions. Interestingly, in addition to decreasing neuronal cell death, LA also inhibited fAβ formation from amyloid β-protein, ameliorating the neurological damage induced by Aβ, and significantly restored AChE activity. This evidence suggests that LA presents a potential role in enhancing cholinergic and cognitive functions. These neuroprotective effects may be related to the properties of LA in ameliorating memory loss associated to neurodegenerative diseases.

Remarkably, LA was able to reverse age-associated glutamatergic NMDA receptor deficits (Stoll et al., 1993), which might be centrally related to LA memory-improving effects. LA was also shown to improve the function of neurotransmitter systems, including dopamine, serotonin, and norepinephrine. Taken together, these findings provide evidence that LA can reverse loss of neurotransmitters, their receptors and responsiveness to neurotransmitters, which can underlie its effects on cognitive functions.

In summary, this review has described and discussed relevant studies investigating the effects of LA on cognition as well as its cellular and molecular effects, aiming to improve the understanding of the therapeutic potential of LA in memory loss during aging and patients suffering from neurodegenerative disorders. Although the mechanisms of action of LA are not fully understood, multiple pathways are likely to be involved in its neuroprotective properties. The memory-improving effects and neuroprotective actions of LA support its use as an adjuvant treatment for neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

PM has performed literature search and has written the first draft of the manuscript. NS has extensively revised and contributed in writing the final version of the manuscript.

FUNDING

This research was supported by the National Council for Scientific and Technological Development (CNPq; grant number 308290/2015-1 to NS).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Pharmacology

ORIGINAL RESEARCH
published: 08 January 2018
doi: 10.3389/fphar.2017.00969

β-Sheet Breaker Peptide-HPYD for the Treatment of Alzheimer's Disease: Primary Studies on Behavioral Test and Transcriptional Profiling

Weiying Liu[1†], Fengxian Sun[2†], Moxin Wan[2], Fang Jiang[2], Xiangyu Bo[3], Laixiang Lin[4], Hua Tang[1] and Shumei Xu[2*]*

[1] Department of Pathogen Biology, Tianjin Life Science Research Center, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China, [2] Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Tianjin Medical University, Tianjin, China, [3] Department of Pathology, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Science and Peking Union Medical College, Tianjin, China, [4] Key Laboratory of Hormone and Development (Ministry of Health), 2011 Collaborative Innovation Center of Tianjin for Medical Epigenetics, Metabolic Diseases Hospital and Tianjin Institute of Endocrinology, Tianjin Medical University, Tianjin, China

OPEN ACCESS

Edited by:
Antonella Gasbarri,
University of L'Aquila, Italy

Reviewed by:
Maria Grazia Morgese,
University of Foggia, Italy
Luigia Trabace,
University of Foggia, Italy

***Correspondence:**
Weiying Liu
liuweiying3@126.com
Shumei Xu
xushm@tijmu.edu.cn

[†] These authors have contributed equally to this work.

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 21 September 2017
Accepted: 19 December 2017
Published: 08 January 2018

Citation:
Liu W, Sun F, Wan M, Jiang F, Bo X, Lin L, Tang H and Xu S (2018) β-Sheet Breaker Peptide-HPYD for the Treatment of Alzheimer's Disease: Primary Studies on Behavioral Test and Transcriptional Profiling. Front. Pharmacol. 8:969. doi: 10.3389/fphar.2017.00969

Background: Alzheimer's disease (AD), is a progressive neurodegenerative disease that is characterized by cognitive loss. Most researchers believe that aggregation and accumulation of β-amyloid peptides (Aβ) in brain cells are the central pathological hallmark of this disease.

Methods: Based on the amyloid hypothesis, a 10 amino acids β-sheet breaker peptide HPYD (His-Lys-Gln-Leu-Pro-Phe-Tyr-Glu-Glu-Asp) was designed according to the structure and sequence of the previous designed peptide H102. Accelerated stability test, thioflavine T (ThT) fluorescence spectral analysis and transmission electron microscopy (TEM) imaging were performed to detect the stability and inhibitory effects on the aggregation of $A\beta_{1-42}$ by H102 and HPYD. FITC-labeled HPYD was first tested to determine whether it could be transferred along the olfactory pathway to the brain after nasal administration to mice. Subsequently, the Morris Water Maze (MWM) test for behavioral analysis was used to investigate the learning and memory ability of APP/PS1 transgenic mice by HPYD. Immunohistochemistry and western blot analysis was performed to determine the role of HPYD on Aβ and APP protein levels. In addition, microarray analysis was used to evaluate the effect of HPYD on gene expression in AD mouse models.

Results: Our *in vitro* results demonstrated that HPYD had enhanced stability and inhibitory effects on $A\beta_{1-42}$ aggregation compared to H102. HPYD could be delivered into the brain through nasal administration and improved the learning and memory ability in APP/PS1 transgenic mouse models by reducing Aβ and APP protein levels. In addition, microarray analyses suggested that several genes related to the inflammatory pathway,

AD and gluco-lipid metabolism were dysregulated and could be restored to almost normal levels after HPYD administration to mice.

Conclusions: Our results demonstrated that HPYD could be a potential therapeutic drug candidate for the treatment of AD.

Keywords: Alzheimer's disease, HPYD, β-amyloid peptides, APP, β-sheet breaker peptide

INTRODUCTION

Alzheimer's disease (AD) is a complex, severe neurodegenerative disorder of the central nervous system, which manifest as progressive cognitive decline and memory impairment in the elderly and presenium individuals. More than 44 million people are currently living with AD and the global health care cost in 2010 was about US$818 billion (Weiner et al., 2013). The incidence of AD has been increasing every year, and by 2050 about 131.5 million people will suffer from dementia if there are no effective therapies (Cummings et al., 2016). Although AD has led to a major social and health care problem, there is still no absolute effective treatment for the disease. Hence, it is critical to develop novel drugs and effective therapies for AD.

The pathological characteristics of AD mainly include massive senile plaque deposits, neurofibrillary tangles as well as selective loss of neurons and synapses in specific brain regions, such as the cerebral cortex and hippocampus (Mattson, 2004; Shen and Kelleher III, 2007). Senile plaques contain extracellular deposits of β-amyloid peptide in its fibrillar form and neurofibrillary tangles, which are composed of hyper phosphorylated tau protein. There are many hypothesis regarding the pathogenesis of AD, such as the cholinergic hypothesis (Francis et al., 1999), amyloid hypothesis (Hardy and Allsop, 1991; Mudher and Lovestone, 2002), tau hypothesis (Goedert et al., 1991; Mudher and Lovestone, 2002) and several other hypotheses (Reisberg et al., 1999; Deane and Zlokovic, 2007). The amyloid hypothesis has become the most accepted mechanistic hypothesis for AD pathogenesis, although some amendments have been made.

β-amyloid peptides (Aβ) are 39–42 amino acid peptide residues that are derived from putative intramembranous processing of amyloid precursor protein (APP) by γ-secretase/PS1 aspartyl protease (Selkoe, 1999; Hardy and Selkoe, 2002). Only a small amount of Aβ is generated by the cleavage of APP by β- or γ-secretase in healthy individuals, while a large amount of APP is metabolized by α- and β-secretase without Aβ generation (Haass et al., 1992; Zhang and Xu, 2007). However, genetic mutations in β- or γ-secretase in AD patients results in increased APP cleavage activity, which subsequently generates large amounts Aβ. Aβ has been shown to aggregate and accumulate abnormally in the brain of AD patients, and extracellular amyloid plaques of Aβ peptides aggregation can trigger a cascade of pathologic events leading to nerve fiber entanglement and neuronal apoptosis (Hardy and Selkoe, 2002; Karran et al., 2011).

Recently, it has been demonstrated that smaller and more soluble aggregates, including a variety of compounds of soluble oligomers, ADDLs (amyloid β-derived diffusible ligands) or protofibrils to be the predominant toxic forms of Aβ (Walsh and Selkoe, 2007; Allsop and Mayes, 2014; Karran and De Strooper, 2016; Selkoe and Hardy, 2016). Compared to fibrillar plaques, Aβ oligomers (AβOs) are considered to be the main mediators of cytotoxicity in AD (Ashe and Aguzzi, 2013). The oligomeric Aβ can initiate the phosphorylation of Src kinase Fyn, asparagine endopeptidase (AEP) and GSK3-β, which then can subsequently induce the hyperphosphorylation of tau protein (Martin et al., 2013; Zhang et al., 2014). AβOs and ADDLs bind to synaptic contacts and cellular membranes more rapidly and with higher affinity than fibrillar Aβ (Aβf). This binding compromises the integrity of intracellular membranes to induce an elevation of intracellular Ca^{2+}, which then results in rapid and massive neuronal cell death (Demuro et al., 2005; Deshpande et al., 2006). In addition, Aβ also could upregulate inflammatory cytokines and increase the nitric oxide release to cause neuroinflammation (Hu et al., 1998). Several studies have demonstrated that Aβ can induce the expression of several inflammatory factors, including CASP1, CASP4, PLA2G4A, and PTPRC (Lee et al., 2011; Zhu et al., 2011; Mehta et al., 2012; Kajiwara et al., 2016). It has been reported that soluble $A\beta_{1-42}$ protofibrils could stimulate microglial production of tumornecrosis factor α (TNFα) to stimulate inflammatory responses (Paranjape et al., 2013). Taking all these observations and studies into consideration, the amyloid hypothesis is recognized as the most prominent theory to explain the pathogenesis of AD.

It is thought that Aβ aggregations are generated due to Aβ clearance deficiencies of γ-secretase (Jarrett et al., 1993; Golde et al., 2000; McGowan et al., 2005). Hence, many secretase inhibitors were developed as the initial small-molecular therapies for AD (De Strooper et al., 2010). Semagacestat, a γ-secretase inhibitor, reached Phase 3 clinical trials, but was halted due to adverse events of worsening cognition and activity, as well as increasing the incidence of skin cancer (Karran and De Strooper, 2016). Many other strategies were also considered for AD, for example, inhibiting Aβ aggregation. Tramiprosate was found to inhibit Aβ aggregation by maintaining Aβ in a non-fibrillar form, thus inhibiting amyloid deposition (Gervais et al., 2007). Tramiprosate could significantly reduce brain amyloid plaque load (∼30%) and the cerebral levels of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ (∼20–30%) in TgCRND8 mice (Gervais et al., 2007). However, no significant therapeutic effects were observed as primary outcomes in the Alzheimer Disease Assessment Scale-cognitive subscale (ADAS-cog) and Clinical Dementia Rating-Sum of Boxes (CDR-Sum of Boxes) in phase III trials (Aisen et al., 2011). Nonetheless, ALZ-801, a novel prodrug of tramiprosate, has shown excellent oral safety and tolerability, and its PK characteristics were significantly improved compared to oral tramiprosate in phase I studies (Hey et al., 2017). Additionally, immunotherapies were considered

for AD therapy. Bapineuzumab and solanezumab represent two humanized monoclonal antibodies that increases the clearance of Aβ by specifically targeting amino acids 1–5 and 16–24 of Aβ peptide, respectively. However, Phase 3 clinical trials of bapineuzumab were halted after the completion of two trials because it did not improve clinical outcomes in patients with AD (Salloway et al., 2014). Similarly, solanezumab also failed to meet the primary outcome in Phase 3 clinical trials (Siemers et al., 2016). Although many amyloidocentric drugs have failed after Phase 3 clinical trials, it does not diminish the pathogenic theory of this disease.

Aβ consists of a hydrophobic carboxyl terminus and a hydrophilic amino terminus. The hydrophobic carboxyl terminus of Aβ mainly consists of β-sheets while the hydrophilic amino terminus mainly consists of α-helix and β-turns (Chou and Fasman, 1977). Aggregation of monomeric Aβ into oligomers are formed through the internalization of the hydrophobic carboxyl terminus and exposing the hydrophilic amino terminus (Hilbich et al., 1992). The carboxy terminus of Aβ is critical for amyloid formation.

β-sheet breaker peptides (also referred to as peptidic inhibitors) are a class of compounds that are highly potent in ameliorating $A\beta_{1-42}$- or α-synuclein-inflicted cell toxicity (Watanabe et al., 2002; El-Agnaf et al., 2004). β-sheet breaker peptides are homologous to regions of the β-sheet hydrophobic carboxyl segments and highly effective in inhibiting Aβ amyloidogenesis (Jarrett et al., 1993). Based on the amino acid residues 17–21 of $A\beta_{1-42}$, we previously designed a β-sheet breaker peptide H102 (His-Lys-Gln-Leu-Pro-Phe-Phe-Glu-Glu-Asp) that can reduce amyloid load and cerebral damage and improve the learning and memory ability of AD animal models (He et al., 2008; Lin et al., 2014). However, H102 was not very stable. Hence, we designed an alternative β-sheet breaker peptide HPYD (His-Lys-Gln-Leu-Pro-Phe-Tyr-Glu-Glu-Asp) by substituting Phe with Tyr. The stability of HPYD and the ability to inhibit Aβ aggregation were studied *in vitro*. HPYD displayed excellent stability and the ability to inhibit Aβ aggregation compared to H102. However, HPYD efficacy for the treatment of AD *in vivo* remained to be elucidated.

In this study, we first compared the stability of H102 and HPYD using the accelerated stability test, and then performed inhibitory studies on the aggregation of $A\beta_{1-42}$. We also investigated the ability of HPYD to transverse into the brain through the olfactory pathway after nasal administration of fluorescein isothiocyanate (FITC)-labeled HPYD. The effect of HPYD on APP/PS1 transgenic mice behavior and the APP and Aβ expression in the brain were also investigated. Furthermore, we profiled the gene expression in normal mice (control group), APP/PS1 transgenic mice (model mice) and APP/PS1 transgenic mice treated with HPYD (HPYD group) using gene microarrays. Gene ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed to annotate their functions. Our findings not only has important implications for the potential treatment of AD using HPYD, but also provide insights to the mechanism of Aβ toxicity in AD patients and for the development of new therapeutic strategies for AD.

MATERIALS AND METHODS

Animals

Nude mice (weight: 26 to 28 g; age: 2 months), APP/PS1 mice (weight: 25.3 to 28.1 g; age: 8 months) and C57/6J mice (weight: 25.5 to 28.6 g) were purchased from the institute of laboratory animal sciences, CAMS & PUMC (Chinese Academy of Medical Sciences and Peking Union Medical College). APP/PS1 mice have been previously demonstrated to form amyloid plaques, which have been approved by institute of laboratory animal sciences, CAMS and PUMC. All animal experiments were performed in accordance with the China Physiological Society "Guiding Principles in the Care and Use of Animals" approved by Tianjin Medical University Animal Care and Use Committee (NO. 20130021).

Compounds

H102, FITC-labeled HPYD and HPYD were synthesized using the Fmoc solid-phase synthesis method and purified by HPLC (Gill Biotechnology Company, Shanghai, China). The compounds were greater than 95% pure as measured by HPLC-MS. HPYD, a polypeptide comprising the amino acid (AA) sequence of His-Lys-Gln-Leu-Pro-Phe-Tyr-Glu-Glu-Asp, was dissolved in normal saline.

Accelerated Stability Test

0.5 mg of HPYD or H102 was dissolved in 1 mL of 0.2 mol/L phosphate buffer containing 100 mg/L of trypsin. The trypsin solution and polypeptide were mixed at a ratio of 1:4, and the solution was placed in a temperature controlled water bath shaker at 37°C for 0, 40, 100, 160, 220, 280, 340, and 400 min. The samples were then heated to 80°C for 10 min, and the stability of HPYD and H102 was detected at the different time points. High performance liquid chromatography (HPLC) analysis of HPYD and H102 was performed using a Phenomerex C18 column (250 mm × 4.6 μm, 5 μm; Sigma, Inc. U.S.A.). The mobile phase consisted of solvent A, 0.1% TFA in acetonitrile and solvent B, 0.1% TFA in water at a ratio of 22.5:77.5 (v/v). The sample injection volume was 20 μL. The flow rate was 1 mL/min, and the detection wavelength was 220 nm.

Thioflavine T (ThT) Fluorescence Spectral Analysis

$A\beta_{1-42}$ freeze-dried powder was dissolved in 50 mmol/L sodium phosphate buffer solution (pH = 7.4) to a concentration of 22.15 μmol/L, and H102 and HPYD were dissolved in the same PBS solution to a concentration of 88.60 μmol/L. $A\beta_{1-42}$ solution was then mixed with H102 and HPYD respectively in equal volumes. The Aβ fibrils were grown at 37°C for 24 hrs. 10 μL solution from each group was then added to 990 μL of 3.0 μmol/L ThT solution and fluorescent intensity was measured using a VARIAN PTC-Au00-01058 fluorescence spectrophotometer (VARIAN, USA) with an excitation wavelength of 453 nm and emission of 478–486 nm.

Transmission Electron Microscopy (TEM)

The $A\beta_{1-42}$ samples (11.07 μmol/L 20 μL) were incubated in 37°C for 5 days, and the mixture of $A\beta_{1-42}$ (22.15 μmol/L 10 μL) with HPYD (88.61 μmol/L 10 μL) or H102 (88.61 μmol/L 10 μL) were incubated under the same conditions for 5 days. Aliquots (5 μL) of each sample was spotted onto thin carbon substrates supported by carbon film on a 300 mesh copper grid for 15 min and blotted dried. The TEM grids were negatively stained with 2% uranyl acetate for 2 min and air dried. The samples were subsequently imaged using a Hitachi H-600 transmission electron microscope.

Fluorescence Imaging System Analysis

Nude mice were anesthetized using 20% urethane (5 mL/kg), and then placed on the VFIS observation platform. Nude mice were then irradiated at 490 nm excitation without treatment, and then irradiated for 5, 15, and 30 min after nasal administration with FITC-HPYD (5.535 mg/kg). Thirty minutes after nasal administration with FITC-HPYD, the mice were euthanized and the hippocampus, cortex, olfactory bulb, heart, lung, liver, spleen and kidneys were removed. These organs were then irradiated at 490 nm excitation.

HPYD Treatment

The APP/PS1 transgenic mice were randomized into the model and HPYD treatment group ($n = 12$ for each group). C57BL/6J mice with the same genetic background and age served as the normal controls. Treatment group received intranasal administration of HPYD saline solution (33 mg/mL, 5 μL/d), while the normal control group and model group were given with the same volume of saline for 30 days.

Behavioral Test

The behavioral test was performed after 30 days of intranasal administration using the Morris Water Maze (MWM) according to our previous report (Lin et al., 2014). Briefly, the MWM test was conducted in a circular pool with a diameter of 80 and 32 cm deep. The pool was filled with 25 ± 1°C water to a depth of 1 cm higher than the escape platform. The water was made opaque white to hide the escape platform. The orientation and navigation experiments were conducted for 5 days to record the time intervals when the animals climbed onto the platform. If animal failed to find the hidden platform within 90 s, the mouse was placed back onto the platform for 20 s, and the escape latency was recorded as 90 s. The platform was placed in the center of the third quadrant and remained in the same position throughout the orientation and navigation experiment. On the 6th day, the platform was removed, and each mouse was allowed to swim freely for 90 s to record the frequency of passing the hidden platform and the original angle.

Immunohistochemistry

At the end of the MWM test, the mice were euthanized and their brains were removed and rapidly placed on ice. The brain tissues were then fixed in 4% paraformaldehyde solution and waxed. Tissue sections were then dewaxed, and antigen retrieval was performed using boiling citrate buffer solution. This was followed by incubating the sections in 3% H_2O_2 solution at room temperature for 10 min to block endogenous peroxidase activity. The sections were then blocked by incubating with normal goat serum at room temperature for 15 min, and subsequently diluted antibodies (Aβ: 1:100; APP: 1:100) were added and incubated overnight at 4°C. Biotin-labeled secondary antibody was added and incubated for 15 min at room temperature, followed by incubation with strept avidin-biotin complex (SABC) and DAB chromogenic reagent. Finally, the sections were counterstained with hematoxylin and observed under a microscope.

Western Blotting

Western blot analysis was performed according to the detail procedures described previously. Primary Aβ antibody (1:200) was purchased from Abcam (USA) and anti-APP antibody (1:200) was purchased from Wuhan BOSTER Bio Company (Wuhan, China). The secondary goat anti-rabbit antibody was purchased from Sigma-Aldrich (St Louis, MO, USA).

Microarray Analysis

Microarray hybridization was carried out by Shanghai GMINIX Biotech Limited Company (China) using the GeneChip® Mouse Gene 1.0 ST Array (Affymetrix, USA Scientific) with 770,317 probes. Briefly, total RNA was extracted from frozen brain tissues using the RNeasy mini kit (Qiagen, Valencia, CA) and used for cDNA synthesis using the cDNA synthesis kit (Affymetrix, Inc., USA). cDNA was labeled using the Gene Chip2 WT Terminal Labeling Kit (Affymetrix, Inc., USA), and then the labeled cDNA was hybridized to the mouse Gene chip at 45°C for 16 h. The Gene chip was then washed with wash solution A, wash solution B and deionized water, and stained using Cocktail 1 and Cocktail 2. The Gene Chip 2 Scanner 300 7G (Affymetrix Inc.) and the AGCC Scan Control software was used for date analysis. The gene ontology (GO) enrichment analysis was performed using the GOEAST software toolkit ($P \leq 0.05$), and signaling pathway analysis was performed using the KEGG data software. Software Matlab 7.1 and java, was used to build and analyze the dynamic Gene networks, and network maps of the differentially expressed genes were constructed from the different states.

Accession Numbers

The Gene Expression Omnibus accession number for normal mice, model mice and HPYD mouse expression profiles is GSE104249.

Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR analysis was performed using an ABI 7500 thermocycler (Applied Biosystems) with UltraSYBR Mixture purchased from Beijing ComWin Biotech Co., Ltd. (Beijing, China). Gene transcript normalization was performed using housekeeping gene β-actin. All primers used for RT and qPCR are listed in **Table 1**.

Statistical Analysis

All experiments were performed at least three times. Data is presented as mean ± SD. The date of escape latency from MWM were analyzed by multivariate analysis of variance (ANOVA) and the other data were analyzed by one-way ANOVA, followed by

TABLE 1 | Primers used in this study.

Name	Sequence (5′-3′)
PLA2G4A-qF	AGAAGGACGTGCCGGAAAGGTGCAT
PLA2G4A-qR	CCGCTGCGTCGAGCTCGTCATCGAA
CLEC4D-qF	TACCACACGAGAGTAACGTGCATCC
CLEC4D-qR	CTCTCATGCCAGGTCTGGTTGTCAT
APP-qF	ACCGGGCCATGCCGCGCAATGATCT
APP-qR	GCCACACACCGCCATGCAGTACTCT
PTPRC-qF	AACTGAGCACAACAGAGAATGCCCT
PTPRC-qR	AGCGTGGATAACACACCTGGATGAT
CDK1-qF	GACTTGAAAGCGAGGAAGAAGGAGT
CDK1-qR	TCAAAGATGAGATACAGCCTGGAGT
CASP1-qF	TCTGGGGTATACCGTGAAAGTGAAA
CASP1-qR	GATACCATGAGACATGAATACAAGG
SCN9A-qF	TGCCCTCATTGAACAACGCATTTCT
SCN9A-qR	TCTCCGTAGATGAAGGGTAGCTGTT
FCGR4-qF	CCCCAAGTGGGTCAGGGTGCTTGAG
FCGR4-qR	TGGGTCACTGATCGTGGAGAGGGCT
CLEC5A-qF	GACGAAAGTACCATGCCTACAAGGA
CLEC5A-qR	TGGAGTGTTGACAATTGCCAGTGTG
DDX3X-qF	AAGGGCGTTATATCCCACCTCATTT
DDX3X-qR	AAGAAGCTAGACTTCCCTCTTGAAT
CASP4-qF	ACGCAGTGACAAGCGTTGGGTTTTT
CASP4-qR	ATTAGCTTCACCATGGTGGCTGCCT
IRGM1-qF	TTTAAGAGAAGGAAAACTACTGGAA
IRGM1-qR	GATGACTCGAAGTGCATTGATGAAA
RSAD2-qF	GAGTGGCCTGGCCATCCTGTTCTGC
RSAD2-qR	TGAAGTGGTAGTTGACACTCACGGG
IFIH1-qF	GTGCTGGACCACCTCATCTTTCTGT
IFIH1-qF	CCACGAACATCTGCGTCCATCCCAG
APOBEC3-qF	TCCACTTTAAGAACCTAGGCTATGC
APOBEC3-qR	CTTAAAGACCCCATGGTGAAGGGAG
NAIP2-qF	TTAGTACTGGCTACTGGAAACTGTC
NAIP2-qR	ATCTTCTCTGCTGAGCTCCAGCCTG
NAIP5-qF	CCAGGAGTCTGAGTGAGCAGCTAAG
NAIP5-qR	GCTGGCTGATGTGCTTTGAAATGAT
IRF7-qF	CCCCAGCCGGTGATCTTTCCCAGTC
IRF7-qR	ACTTGCCCATACGCAGGGCCCACAG
β-actin-qF	CGTGACATTAAGGAGAAGCTG
β-actin-qR	CTAGAAGCATTTGCGGTGGAC

Student-Newman-Keuls test. All the analysis was performed by SPSS statistical software (version 21, IBM, Armonk, NY). $P \leq 0.05$ was considered to be statistically significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

RESULTS

HPYD had a Better *in Vitro* Stability and Inhibitory Effect on Aggregation of $A\beta_{1-42}$ Compared to H102

To investigate the stability of HPYD *in vitro*, accelerated stability test using trypsin was performed. After treatment with 100 mg/L trypsin at 37°C for 400 min, followed by heat treatment for 10 min at 80°C, the concentration of HPYD was 81.67% ± 0.42, while the concentration of H102 was only 41.07% ± 0.17, indicating that the stability of HPYD was better compared to H102 (**Table 2**). In addition, to compare the inhibitory effects on aggregation of $A\beta_{1-42}$ with H102, ThT fluorescence spectral analysis and TEM was performed. Results showed that HPYD could significantly inhibit the aggregation of $A\beta_{42}$ compared to H102 (**Figures 1A,B**). Taken together, this demonstrates that HPYD had better *in vitro* stability and inhibitory effects on $A\beta_{1-42}$ aggregation compared to H102.

Uptake of FITC-HPYD Into the Brain after Nasal Administration

HPYD, a β-sheet breaker peptide of 10 AAs, is difficult to administer orally or intravenous because it easily undergoes rapid *in vivo* degradation in the gastrointestinal tract and blood. Therefore, to determine whether HPYD can be administered to the brain through the olfactory pathway after nasal administration, HPYD was labeled with FITC and observed by fluorescence imagining of the brain. Before nasal administration, nude mice were irradiated at 490 nm excitation wavelength, and then were subsequently administrated with FITC-HPYD though nasal cavity and irradiated after 5, 15 and 30 min with wavelength of 490 nm. Results showed that FITC-HPYD first appeared at the brain area at 5 min after nasal administration, and the fluorescence gradually expanded with time. As shown in **Figure 2A**, 30 min after nasal administration, the fluorescence had spread over the whole body of the nude mouse, however, the fluorescence intensity in the brain was the strongest compared to other organs. The mice were then euthanized and the hippocampus, cortex, olfactory bulb, heart, lungs, liver, spleen and kidneys were removed at 30 min after nasal administration and irradiation. The results indicated that the fluorescence intensity of the olfactory bulb was the strongest, with the cortex and the hippocampus having the second and third strongest intensities, respectively. The other major organs showed varying degrees of fluorescence intensity. Among these organs, the lungs had the strongest intensity, followed by the liver (**Figure 2B**). These results demonstrated that FITC-HPYD could be delivered into the brain through nasal administration.

HPYD Improves the Learning and Memory Ability in APP/PS1 Transgenic Mice

MWM test for behavioral analysis is a common strategy to investigate the learning and memory ability in AD mouse models. To determine the efficacy of HPYD on APP/PS1 transgenic mice, the MWM test was conducted for 6 days. First, the escape latency test was performed from Day 1 to Day 5. As shown in **Figures 3A,B**, the control group and the HPYD group displayed no significant differences from Day 1 to Day 5. In addition, the control group vs. the model group, or the model group vs. the HPYD group, showed no significant difference from Day 1 to the Day 3. However, significant differences were observed between the HPYD group and the model group at Day 4, and in addition, statistical significant differences between the model group and control group were observed on Day 4. The

TABLE 2 | The stability of H102 and HPYD by accelerated stability test.

Variable	H102	HPYD
Initial concentration, mg/L	400 ± 0.20	400 ± 1.21
Study time (min)[a]		
0	100 ± 1.28	100 ± 0.55
40	90.01 ± 0.78	96.26 ± 1.13
100	77.92 ± 0.69	91.98 ± 0.36
160	70.62 ± 0.31	91.04 ± 0.62
220	60.46 ± 0.51	91.14 ± 0.83
280	56.26 ± 0.28	85.27 ± 0.57
340	49.60 ± 0.45	81.11 ± 0.96
400	41.07 ± 0.17	81.67 ± 0.42

[a]*Percentage of initial concentration (Mean ±_SD [%]; n = 3) of H102 (400 mg/L) and HPYD (400 mg/L) remaining after treatment with 100 mg/L trypsin at 37°C for 400 min, followed by heat treatment for 10 min at 80°C.*

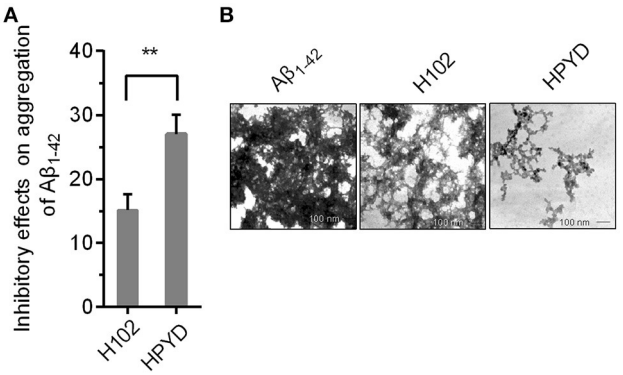

FIGURE 1 | Inhibitory effect of HPYD and H102 on $A\beta_{1-42}$ aggregation. **(A)** ThT fluorescence spectral analysis of the inhibitory effects of HPYD and H102 on $A\beta_{1-42}$ aggregation. **(B)** TEM observations of the inhibitory effects of HPYD and H102 on $A\beta_{1-42}$ aggregation. All of the experiments tests were repeated in triplicates. Statistical significance was denoted with; $^{**}p < 0.01$.

escape latency test indicated that the APP/PS1 transgenic mice, normal mice and the HPYD group mice all required time to find the platform at the start of the test, however, the learning and memory ability demonstrated significant differences after treatment with HPYD in APP/PS1 transgenic mice after 3 days of training. On the 6 day, spatial probe test was performed on the mice without the platform. As shown in **Figures 3C–E**, memory ability was estimated by the frequency passed the hidden platform (**Figure 3C**) and original angle (**Figure 3D**). Frequency passed the hidden platform in the target quadrant displayed significant differences between the model group and the control group, indicating that the model group had a poor ability to find the platform compared to the control group. However, the HPYD group demonstrated an improved ability to find the platform compared to the model group. No significant differences were observed between the HPYD group and the control group. Finally, the ability to locate the original angle was also investigated at the 6th day. The HPYD group was able to find the location of the original angle much quicker compared to the model group, indicating that the HPYD group mice had relatively good memory. No significant differences were observed between the control group and the HPYD group. Collectively, these results indicate that HPYD could improve the learning and memory ability in APP/PS1 transgenic mice after nasal administration of HPYD.

HPYD Reduces Aβ and APP Protein Levels in APP/PS1 Transgenic Mice

HPYD is a novel β-sheet breaker peptide that could inhibit $A\beta_{1-42}$ aggregation *in vitro*. To determine the effect of HPYD on Aβ *in vivo*, HPYD was administrated though the nasal cavity into the brain. Tissues were then harvested after 6 days for immunohistochemistry and western blot analysis. As shown in **Figure 4A**, the hippocampus CA1 and cortex had higher expression of Aβ in the model group mice compared to control group mice, while Aβ was lower in the HPYD group compared to the model group. No significant differences were observed between the control group and the HPYD group. In addition, western blot analysis showed that Aβ was highly expressed in the hippocampus and cortex of the model group mice compared with the control group, while Aβ was expressed at a lower level in the HPYD group mice compared to the model group mice (**Figure 4B**). There were no statistical significant differences between the HPYD group mice and control group mice. APP, the precursor of Aβ, is upregulated in the brain tissues of AD patients. To determine whether HPYD had an effect on APP expression, we determined the expression of APP using immunohistochemistry and western blot (**Figures 4B,C**). Results showed that APP was upregulated in the brain tissues of the model group mice compared to the control group mice, while it was downregulated in the HPYD group mice compared with the model group mice. The expression of APP in the HPYD group mice was similar to the control group. These results indicate that HPYD can reduce Aβ and APP protein levels in APP/PS1 transgenic mice.

Transcriptional Profiling of Brain Tissues from APP/PS1 Transgenic Mice after Treatment with HPYD

To investigate the effect of HPYD on gene expression of APP/PS1 transgenic mice, the brain tissues of the control group, model group and HPYD group mice were harvested at the termination of the MWM test. Total RNA from brain tissues were isolated and microarray analysis was performed by Shanghai GMINIX Biotech Limited. Based on microarray analysis, 119 genes with significant differences were selected for further analysis (**Figure 5A**). Among the selected genes, 15 genes were markedly down-regulated in the model group compared to the control group, however, the expression levels of these genes were restored to normal levels after treatment with HPYD. Additionally, the majority of the selected genes were up-regulated in the model group compared to the control group, which were restored to normal levels after treatment with HPYD. These differentially expressed genes were then analyzed

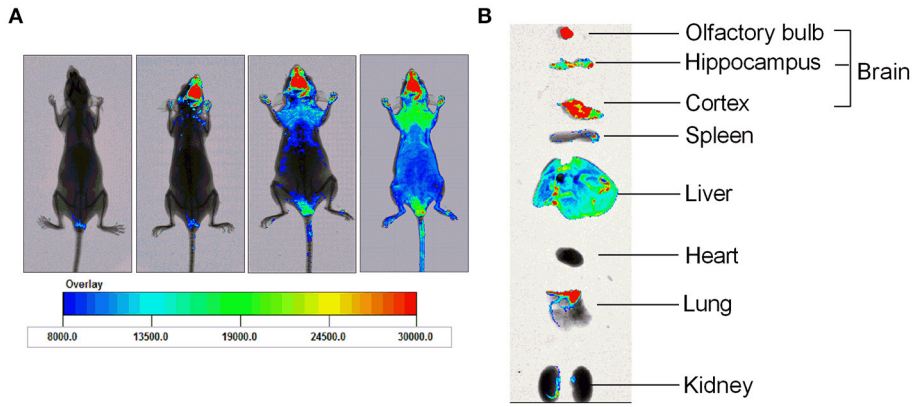

FIGURE 2 | FITC-HPYD transported into brain tissues through nasal administration. **(A)** The *in vivo* distribution of FITC-HPYD at different time points after nasal administration. **(B)** The fluorescence intensity of harvested major organs 30 min after nasal administration.

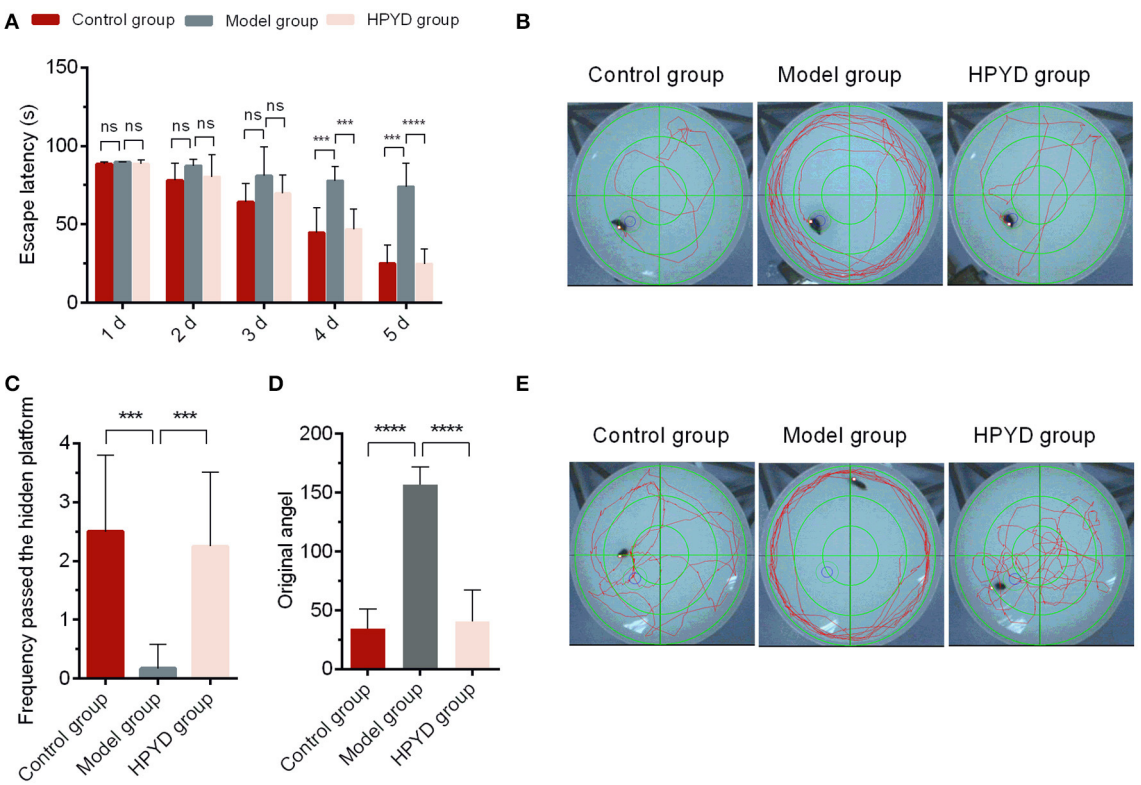

FIGURE 3 | Pathways and behavior of mice in the probe trial. **(A)** Escape latency of mice in the three groups from day 1 to 5. **(B)** The escape-latency of mice obtained through the image capture system of Morris Water Maze test. **(C)** Frequency passed the hidden platform and **(D)** original angle data obtained from the image capture system of the Morris Water Maze test at the Day 6. **(E)** Photographs of spatial probe process obtained from the image capture system of the Morris Water Maze test at Day 6. All of the tests were repeated in triplicates. Statistical significance was denoted with; ***$p < 0.001$; ****$p < 0.0001$; NS, not significant.

by GO analysis to predict their function. The most enriched GO terms are shown in **Figure 5B**, and includes; protein binding, extracellular exosome, immune system, inflammatory reaction and gluco-lipid metabolism. KEGG pathway analysis showed that these genes were involved in many inflammatory response pathways, including viral carcinogenesis, RIG-I-like receptor signaling and NOD-like receptor (NLR) signaling pathway (**Figures 5C,D**). Co-expression network analysis revealed gene-function relationships of the differential gene expressions, including CDK1, PLA2G4A, APP and SCN9A, which were reported to be involved in AD (Ling et al., 2003; Schaeffer et al., 2010; Hilgeroth et al., 2014). In addition, many

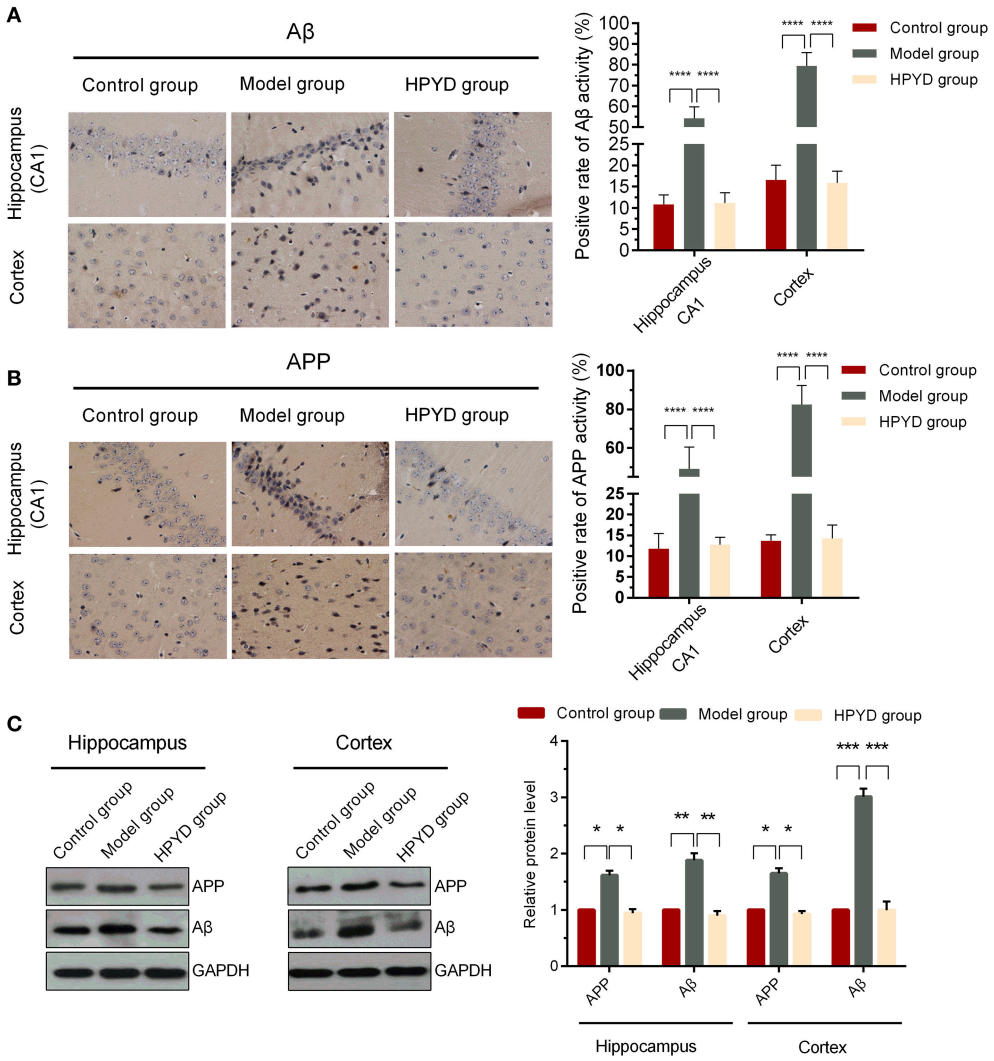

FIGURE 4 | Expression levels of Aβ and APP in the hippocampal CA1 area and cortex. **(A,B)** IHC was performed to determine the expression of Aβ and APP. **(C)** Western blot was used to determine the expression of Aβ and APP. All experiments were repeated in triplicates. Statistical significance was denoted with; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

inflammatory related genes, such as CLEC4D and CLEC5A were differentially expressed. These two genes are members of the C-type lectin/C-type lectin-like domain (CTL/CTLD) superfamily that play important roles in inflammation and immune response (Wu et al., 2013; Wilson et al., 2015). Other genes of interest include; Inflammasome-related CASP1 (encodes caspase-1) and CASP4 (encodes caspase-4) that mediates non-canonical activation of the NLRP3 inflammasome (Schmid-Burgk et al., 2015). NAIP2 and NAIP5, belong to the NAIP/NLRC4 inflammasomes, and play important physiological roles in antibacterial defense and inflammation (Diebolder et al., 2015; Zhang et al., 2015). In addition, 18 genes associated with AD or inflammatory responses were selected for validation by RT-qPCR. There was a good concordance in the expression levels of these genes by RT-qPCR and microarray (**Figure 6**). Taken together, the above results indicated that dysregulated expression of several genes in the model group mice could be restored to normal levels after treatment with HPYD, indicating that HPYD may be a potential therapeutic candidate for treatment of AD.

DISCUSSION

Alzheimer's disease is the most common dementing disease worldwide. Over the past decade, several potential therapeutics were evaluated in clinical trials, but only five drugs (Memantine and four cholinesterase inhibitors) have been approved worldwide to treat AD (Cummings et al., 2016). However, due to the modest clinical efficacy on temporarily ameliorating memory and thought, these five drugs are not very efficacious in treating the underlying cause of AD and preventing the rate of cognitive

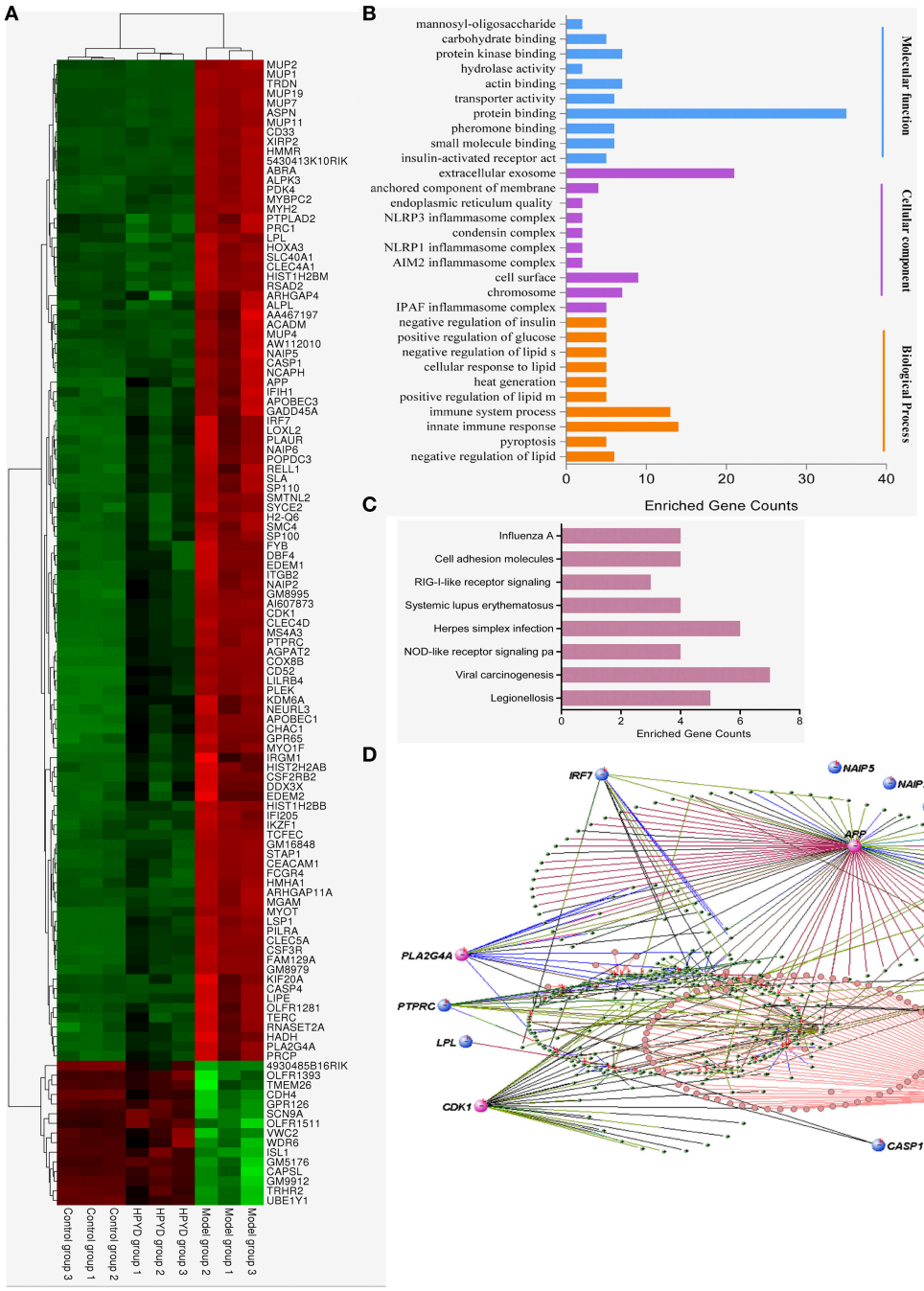

FIGURE 5 | Gene expression profiles after treatment with HPYD. **(A)** Hierarchical clustering of the expression profiles of significantly changed genes after treatment with HPYD. **(B)** The GO terms for molecular functions, cellular components and biological processes were enriched among the significantly changed genes. **(C)** Enriched pathways among the significantly changed genes. **(D)** Co-expression network-based inference of gene-function relationship, node size indicates the node's degree, pink nodes represent genes involve in AD, blue nodes represent genes involve in inflammatory pathway.

decline. Therefore, it is critical to develop novel drugs for the treatment of AD.

The amyloid hypothesis is one of the most accepted mechanistic hypothesis for AD posits that dysregulated aggregation and accumulation of Aβ in the brain leads to nerve fiber entanglement and neuronal apoptosis (Hardy and Selkoe, 2002). The amyloid hypothesis implies that decreasing the levels of Aβ or inhibiting Aβ aggregation could lead to a cure for AD. Based on this hypothesis, many drugs were developed for the treatment of AD. Tramiprosate, a drug of potential

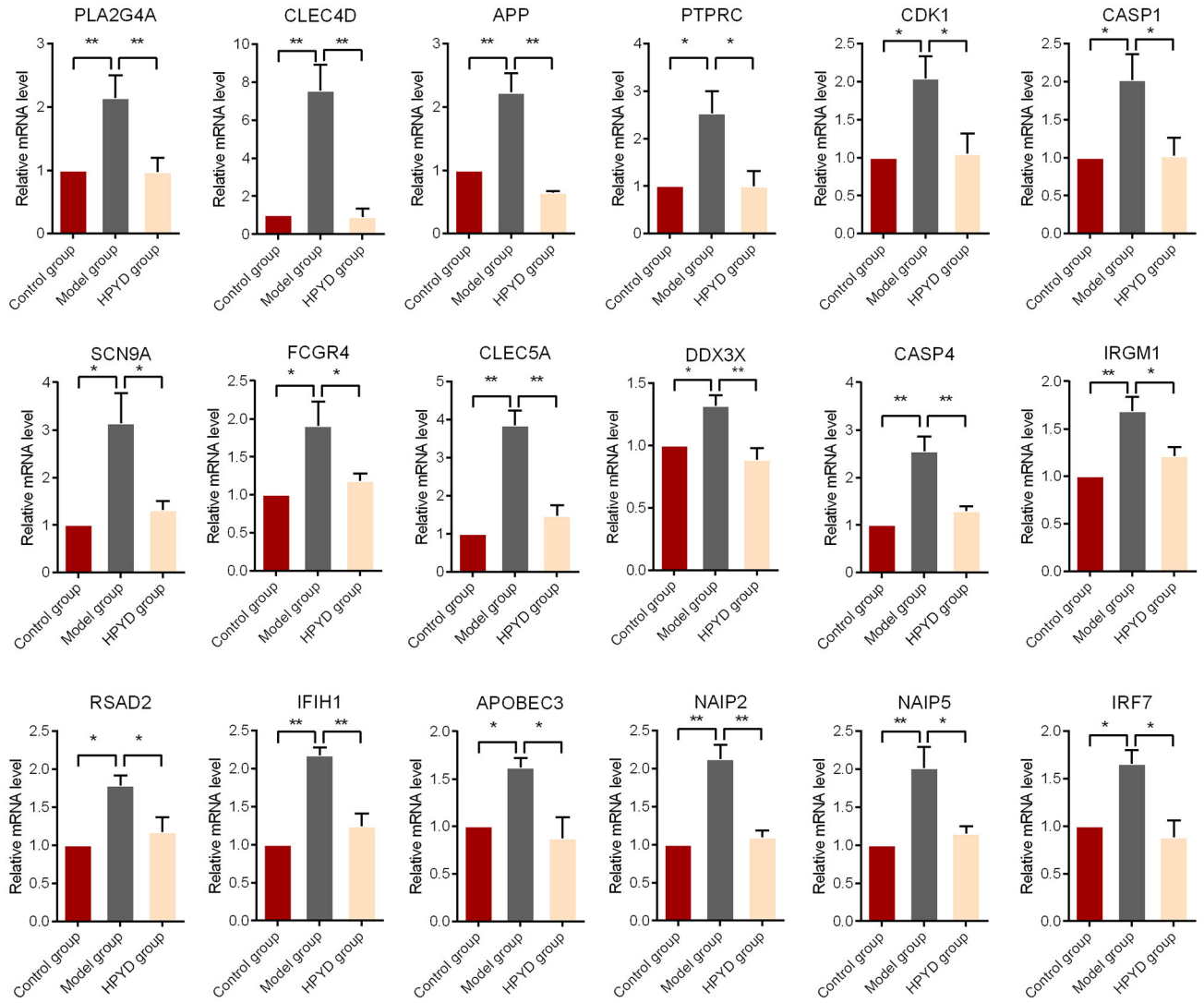

FIGURE 6 | RT-qPCR on the transcriptional levels of 18 selected genes from **Figure 5D**. All of the experiments tests were repeated in triplicates. Statistical significance was denoted with; $^{*}p < 0.05$; $^{**}p < 0.01$.

interest for the treatment of AD, inhibits Aβ aggregation and amyloid deposition (Gervais et al., 2007). Bapineuzumab and solanezumab, two humanized monoclonal antibodies that specifically target amino acids 1–5 or 16–24 of Aβ peptide, increases the clearance of Aβ and decreases amyloid deposition in the brain (Salloway et al., 2014). Despite efficacy in AD animal models, the above mentioned drugs all failed in Phase 3 clinical trials due to efficacy. However, the failure of several amyloidocentric drugs for the treatment of AD did not diminish the pathogenic theory of this disease. Numerous novel drugs have been designed or screened for inhibiting Aβ production. For example, Icariside II (ICS II), an anti-cancer natural compound extracted from *Herba Epimedii* Maxim, was demonstrated to inhibit Aβ production by reducing APP and BACE1 expression in APP/PS1 transgenic mice (Yan et al., 2017). ALZ-801, a novel prodrug of tramiprosate, has been shown to have excellent oral safety and tolerability compared to tramiprosate though phase I studies (Hey et al., 2017).

Previously, we designed a β-sheet breaker peptide, H102, that could inhibit Aβ aggregation as well as reducing amyloid load in the brains of APP/PS1 transgenic mice, thus limiting brain damage and improving the symptoms of AD in animal models (He et al., 2008; Lin et al., 2014). However, H102 was not stable *in vitro*. We designed alternative β-sheet breaker peptide, HPYD, by substituting Phe7 with Tyr7. This peptide showed higher *in vitro* stability and better efficacy of inhibiting Aβ aggregation compared to H102. In addition, FITC-labeled HPYD demonstrated that the peptide could enter the brain after nasal administration. MWM test demonstrated significant spatial learning and memory disorders in APP/PS1 transgenic model mice, which were in concordance with previous studies (Yan et al., 2017). Several studies have also demonstrated that Aβ

and APP protein expression levels were markedly increased in APP/PS1 transgenic model mice. These outcomes were effectively reversed by treatment with HPYD through nasal administration. Our results indicate that HPYD may be a potential therapeutic candidate for the treatment of AD.

To further demonstrate that HPYD is a potential therapeutic for AD, we analyzed the mRNA levels of APP/PS1 transgenic mice to investigate the effect of HPYD on the brain. APP/PS1 transgenic model mice have early memory dysfunctions even before the degeneration of synapses and neurons (Dickey et al., 2003). The mRNA levels of many genes in APP/PS1 transgenic model mice are dysregulated, but are restored to normal levels after treatment with HPYD. The 119 genes that were differentially expressed were involved in inflammatory reaction, gluco-lipid metabolism and other pathways. Neuro-inflammation has been recognized as playing an important role in the pathogenesis of AD (Cacquevel et al., 2004; Sagy-Bross et al., 2013), which is mediated by microglia (MG) that can participate in the immune response, leading to increase in pro-inflammatory cytokines and chemokines (Lucin and Wyss-Coray, 2009; Prinz et al., 2011). Inflammasomes are responsible for the maturation and release of pro-inflammatory cytokines and the activation of an inflammatory form of cell death. NLRP3 inflammasome, one of the most widely studied members of the NLR family, can be activated by Aβ and enhances AD progression by mediating a detrimental chronic inflammatory tissue response (Heneka et al., 2013). Our results demonstrated that inflammasome-related CASP1 and CASP4, which mediates non-canonical activation of the NLRP3 inflammasome, was highly expressed in APP/PS1 transgenic model mice, which was in concordance with previous studies (Schmid-Burgk et al., 2015). Inflammatory factors, like APOBEC3, CLEC4D, DDX3X, FCGR4, IFIH1, IRF7, NAIP2, NAIP5, CLEC5A, PTPRC and PLA2G4A, were also overexpressed in APP/PS1 transgenic model mice. PLA2G4A has been demonstrated to mediate apoptotic neuronal death in AD brain and could be induced by aggregated Aβ peptide$_{1-42}$ (Sagy-Bross et al., 2013). PTPRC (CD45) was upregulated in microglial and has been association with AD (Masliah et al., 1991). In addition, we also observed that cell-cycle protein CDK1 (CDC2) was upregulated in APP/PS1 transgenic model mice, which is associated with the pathogenesis of AD (Johansson et al., 2005). Most importantly, our study also found that the disordered changes in neurons were effectively restored by treatment with HPYD through nasal administration, indicating HPYD as a potential therapeutic for AD.

In summary, we designed a new β-sheet breaker peptide HPYD, which showed better *in vitro* stability and inhibitory effects on Aβ$_{1-42}$ aggregation compared to H102. HPYD could improve the learning and memory ability in APP/PS1 transgenic mice by reducing Aβ and APP protein levels. Microarray analyses demonstrated that dysregulated gene expression in model mice could be restored to normal levels after treatment with HPYD by inhibiting the aggregation of Aβ. This provides a novel therapeutic strategy for the treatment of AD.

AUTHOR CONTRIBUTIONS

SX conceived project, WL, FS, and SX designed the study and wrote the paper. WL and FS performed the experiments. MW, FJ, XB, and LL provided technical assistance and contributed to the preparation of the figures and manuscript. HT contributed to English editing and academic writing in the whole manuscript. All authors reviewed the results and approved the final version of the manuscript.

FUNDING

This work was supported by grants from Tianjin science and technology plan projects (no: 16YFZCSY01000), National Natural Science Foundation of China (no: 81602512) and China Postdoctoral Science Foundation (no: 2015M581307).

ACKNOWLEDGMENTS

We are grateful to Miss Qing Li from Tianjin cancer institute & hospital for her relevant critical revision of this manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 11 January 2018
doi: 10.3389/fnins.2017.00757

Frontal Control Process in Intentional Forgetting: Electrophysiological Evidence

Heming Gao, Mingming Qi and Qi Zhang*

School of Psychology, Liaoning Normal University, Dalian, China

In this study, we aimed to seek for the neural evidence of the inhibition control process in directed forgetting (DF). We adopted a modified item-method DF paradigm, in which four kinds of cues were involved. In some trials, the words were followed by only a forgetting (F) cue. In the other trials, after a word was presented, a maintenance (M) cue was presented, followed by an explicit remembering (M-R) cue or an forgetting (M-F) cue. Data from 19 healthy adult participants showed that, (1) compared with the remembering cue (i.e., M-R cue), forgetting cues (i.e., M-F cue and F cue) evoked enhanced frontal N2 and reduced parietal P3 and late positive complex (LPC) components, indicating that the forgetting cues might trigger a more intensive cognitive control process and that fewer amounts of cognitive resources were recruited for the further rehearsal process. (2) Both the M cue and the F cue evoked enhanced N2 and decreased P3 and LPC components than the M-R or M-F cue. These results might indicate that compared with the M-R and M-F cues, both the M and F cues evoked a more intensive cognitive control process and decreased attentional resource allocation process. (3) The F cue evoked a decreased P2 component and an enhanced N2 component relative to the other cues (i.e., M-R, M-F, M), indicating that the F cue received fewer amounts of attentional resources and evoked a more intensive cognitive control process. Taken together, forgetting cues were associated with enhanced N2 activity relative to the maintenance rehearsal process or the remembering process, suggesting an enhanced cognitive control process under DF. This cognitive control process might reflect the role of inhibition in DF as attempting to suppress the ongoing encoding.

Keywords: directed forgetting, maintenance rehearsal, cognitive control, P2, N2

OPEN ACCESS

Edited by:
Antonella Gasbarri,
University of L'Aquila, Italy

Reviewed by:
Alexandra Key,
Vanderbilt University Medical Center,
United States
Alfredo Meneses,
Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico

****Correspondence:***
Heming Gao
siwengaohe@163.com

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *10 September 2017*
Accepted: *29 December 2017*
Published: *11 January 2018*

Citation:
Gao H, Qi M and Zhang Q (2018) Frontal Control Process in Intentional Forgetting: Electrophysiological Evidence. Front. Neurosci. 11:757. doi: 10.3389/fnins.2017.00757

INTRODUCTION

Intentionally ignoring or forgetting out-of-date information is essential for memory function (Anderson et al., 2004; Anderson and Hanslmayr, 2014). The processing of task-relevant information may be disrupted by irrelevant information. Intentional forgetting might be helpful in reducing this interference (Nowicka et al., 2011; Benoit and Anderson, 2012). Intentional forgetting is usually investigated by adopting an item-method directed forgetting (DF) paradigm. During the study phase, remembering or forgetting cues are provided randomly following each item. To-be-remembered (TBR) items are followed by remembering cues, and to-be-forgotten (TBF) items are followed by forgetting cues. Generally, TBR items show superior memory performance over TBF items (Bjork and Woodward, 1973; Basden et al., 1993). This effect is called the DF effect.

According to the selective rehearsal account, the DF effect is due to the selective rehearsal of TBR words (Basden et al., 1993; Sheard and MacLeod, 2005). If a remembering instruction is received, participants engage in an elaborate rehearsal. Successful intentional forgetting occurs owing to the passive decay of an unrehearsed memory trace (Bjork and Woodward, 1973; Basden et al., 1993; MacLeod, 1999). The attentional inhibition account argues that an active inhibition process is triggered by forgetting cues (Geiselman and Bagheri, 1985; Zacks et al., 1996). This inhibitory process might serve to cease the rehearsal process of TBF items or suppress the memory representation (van Hooff and Ford, 2011).

With the merit of high temporal resolution, the event-related potential (ERP) technique has been employed to explore the neural activity underlying DF (Paz-Caballero et al., 2004; van Hooff and Ford, 2011; Gao et al., 2016a). The P2 component has been associated with attentional allocation process, with enhanced attention resulting in increased P2 amplitudes (Thorpe et al., 1996; Bergström et al., 2007; Qi et al., 2016). Some DF studies found that a more positive frontal P2 component was evoked for remembering vs. forgetting cues (Cheng et al., 2012; Gao et al., 2016a), indicating that forgetting cues received fewer amounts of attentional resources. Some studies found that compared with remembering cues, forgetting cues evoked more positive ERPs over the frontal scalp but evoked less positive ERPs over the parietal scalp (Paz-Caballero et al., 2004; van Hooff and Ford, 2011). Recently, some studies found that forgetting cues evoked a more negative N2 over the frontal scalp but decreased P3 and late positive complex (LPC)components over the parietal scalp compared with remembering cues (Yang et al., 2012; Patrick et al., 2015; Gao et al., 2016a). These studies suggested that the enhanced frontal ERP activity associated with forgetting cues might reflect the attentional inhibition process.

In the typical item-method DF paradigm, participants are aware that each item has an equal possibility of being followed by a remembering or a forgetting cue. They do not engage in an elaborate rehearsal when the items are presented. These items are kept in working memory by rote rehearsal or maintenance rehearsal before cues are presented (Woodward et al., 1973; Greene, 1987). Therefore, different cognitive strategies might be adopted in response to different cues. Specifically, remembering cues trigger the elaborate rehearsal process for TBR items, while this process was absent for forgetting trials, in which TBF items are passively decayed or inhibited (Wylie et al., 2008). The ERP cue effect (remembering vs. forgetting cue) might reflect that participants adopted different cognitive strategies. There is no firm evidence showing that forgetting cues trigger the inhibition control process.

Previous studies have demonstrated that cognitive control over overt behavior is always associated with the activity of the frontal/prefrontal cortex. For example, this frontal activity was always observed in motor response inhibition tasks (e.g., Bokura et al., 2001; Aron et al., 2014) and switching tasks (e.g., Dove et al., 2000; Philipp et al., 2013). Goal-directed cognition generally requires cognitive control, and Anderson et al. (2004) suggested that an explicit forgetting instruction might place demands on controlled attention. Conway and Fthenaki (2003) found that the DF effect was diminished in patients with frontal lobe damage. Therefore, it seems that DF may involve the attentional/cognitive control process. Wylie et al. (2008) found that the neural activity associated with intentional forgetting differed from that associated with unintentional forgetting and intentional remembering, and they speculated that the maintenance rehearsal process is associated with the activity of the inferior frontal regions, and that the activity in the parahippocampal area may reflect the attempt to relate the maintained items to the remembering/forgetting instructions. However, no studies have investigated the neural activity associated with the maintenance rehearsal process in DF.

The present study focused on the neural activity of the maintenance rehearsal process. In the item-method DF procedure, the maintenance rehearsal process was interrupted by remembering and forgetting cues. According to the selective rehearsal account, maintenance rehearsal was passively ceased without any further cognitive processes acting upon the TBF information. However, the attentional inhibition account suggested that processing resources were actively withdrawn from the memory representation of TBF items, and attention was inhibited from returning to the memory representations of TBF items. Therefore, if DF is a passive process, decreased neural activity would be found for the forgetting process vs. the maintenance rehearsal process. On the contrary, if enhanced neural activity was found for the forgetting process vs. the maintenance rehearsal process, it might imply that the forgetting cues triggered an active inhibitory process.

In this study, we adopted a modified item-method DF paradigm, in which a maintenance (M) cue was presented before the remembering/forgetting cues (**Figures 1A,B**). Specifically, when participants saw the M cue, they could not know whether the word was TBR or TBF until the following cues (remembering M-R or forgetting M-F) appeared. Therefore, when the M cue was presented, the participants refreshed the words through maintenance rehearsal until the remembering/forgetting cue was presented. The M cues would trigger the maintenance rehearsal process. In the other trials, the word was followed by only a forgetting cue (F), and this word was categorized as a TBF item (**Figure 1C**). The F cues would trigger the DF process. By using this modified paradigm, we investigated the neural activity underlying maintenance rehearsal and DF (i.e., ERPs evoked by M and F cues).

Previous DF studies found that forgetting cues evoked more negative frontal N2 and less positive parietal P3 components compared with remembering cues (Patrick et al., 2015; Gao et al., 2016a,b). A similar ERP effect would be expected for the M-R vs. M-F cues in this study. As a subcomponent of the P3 component, P3a exhibits a fronto-central scalp distribution and has been associated with the reallocation of attentional resources. Therefore, an enhanced P3a would be found for the M-R cue relative to the M-F cue. In addition, it is necessary to note that the M cues always preceded the M-F/M-R cues, and encoding of item information might benefit from maintenance rehearsal, leading to greater memory trace strength for the M-R condition relative

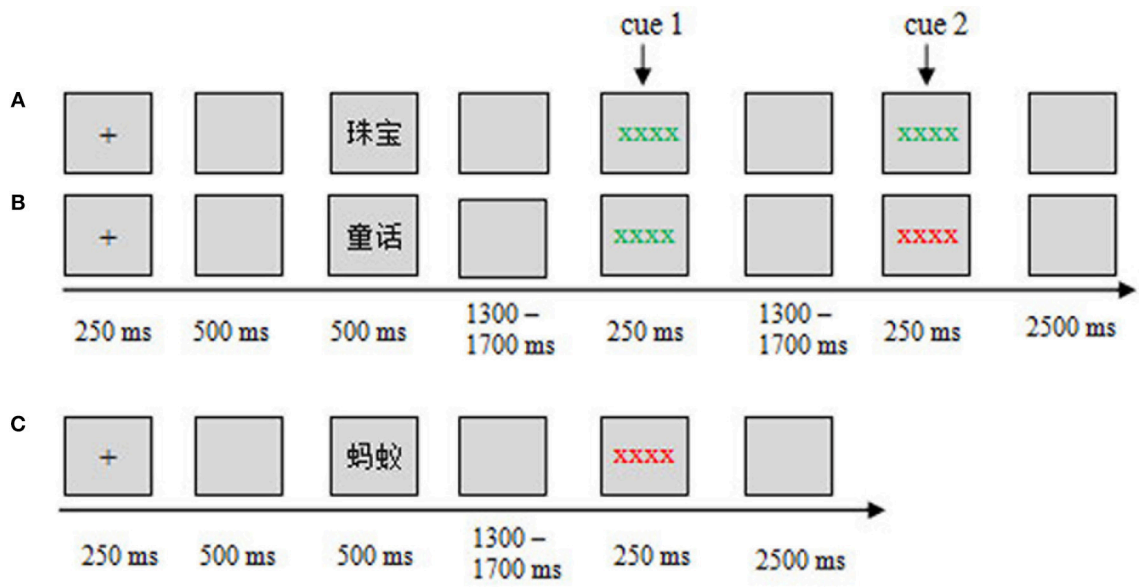

FIGURE 1 | Experimental design and procedure. The sequence of events in the study phase for different conditions is shown: **(A)** M-R, **(B)** M-F, and **(C)** F. ERPs were time-locked to the cue onset.

to the M-F condition. Therefore, we speculated that the ERP difference between the M and M-F cues might be modulated by the differential memory trace strength of the words. To eliminate this order effect, the third condition (F) was designed.

This study mainly focused on the ERP differences between the M and F cues. Previous DF studies found that forgetting cue evoked a decreased frontal P2 component compared with remembering cue (Cheng et al., 2012; Gao et al., 2016a). Similarly, in this study, we predicted that an enhanced P2 component would be observed for the M cues relative to the F cues. We hypothesized that if the memory representation of TBF words was passively decayed, the maintenance rehearsal process would be decreased after the F cues were presented. Therefore, the maintenance rehearsal process triggered by M cues would be more intensive than that triggered by F cues. The parietal P3 and LPC components are associated with the memory rehearsal process (Patrick et al., 2015; Gao et al., 2016b). Accordingly, enhanced P3 and LPC activities would be found for the M cues relative to the F cues. However, if the F cues triggered an active inhibition process to the memory representation of TBF words, the forgetting process might be more effortful than the maintenance rehearsal process. The frontal N2 component is known to be related to executive control (Espinet et al., 2012), inhibitory control of task-irrelevant information (Getzmann et al., 2015; Iannaccone et al., 2015), and the inhibitory process for TBF items (Bergström et al., 2007; Mecklinger et al., 2009; Patrick et al., 2015; Gao et al., 2016a,b). Levy and Anderson (2008) suggested that mechanisms engaged in attentional inhibition might be relate to cognitive control processes that are similar to those used to control overt behavior. Therefore, an enhanced frontal N2 component would be evoked for the F cues relative to the M cues in this study.

MATERIALS AND METHODS

Participants

Twenty undergraduate native Chinese college students took part in this experiment. Because of excessive artifacts in the electroencephalographic recording, one participant was excluded from the analysis (<50% trials were valid after artifact rejection). Therefore, data from 19 participants were included in the analyses (nine male and 10 female participants, mean age = 23.1 years, standard deviation = 1.85). All participants were right-handed and self-reported as healthy. All participants had normal or corrected-to-normal eyesight, and none were color blind. This study was approved by the Research Ethics Committee of Liaoning Normal University of China and was in accordance with the ethical guidelines of the Declaration of Helsinki. All participants have granted their written informed consent, and were paid on completion of the experiment.

Design and Materials

In the study phase, after the words were presented, the participants either received a maintenance cue followed by a remembering/forgetting cue (**Figures 1A,B**) or received only a forgetting cue (**Figure 1C**). Specifically, if a green cue (string of green Xs, cue 1) followed the word, the participants needed to see the following cue (cue 2) to judge this word as TBR or not. If a green cue followed (cue 2), it was a TBR word (**Figure 1A**); if a red cue followed (cue 2), it was a TBF word (**Figure 1B**). If a word was only followed by a red cue (cue 1), this word was a TBF word (**Figure 1C**), and no additional cues followed. Therefore, there were three conditions (three kinds of words): maintain-remember (M-R), maintain-forget (M-F), and forget (F). A within-subject design was used in this study. The three conditions were presented in a pseudo-randomized order, with

the constraint that no more than three consecutive trials could be from the same condition. The assignment of color to the remembering cue or forgetting cues was counterbalanced across subjects.

The learning materials were Chinese double-character nouns, which were selected from the top 8000 words in "The Modern Chinese Frequency Dictionary" with a mean frequency of 7.144 per thousand. Words were assigned to six lists, each containing 60 words. The mean number of strokes and frequency of the words were matched across different lists. Half the lists were used as learning materials for the study phase, and the remaining lists served as new words (distractors) for the test phase. Additionally, two buffer words followed by remembering cues were presented at the beginning and end of the study phase, which were excluded from subsequent analyses. Except for the buffer words, 180 words (60 words per condition) were presented in the study phase. The test phase consisted of 180 old words (60 of each from the M-R, M-F, and F words) and 180 new words (distractors). The sequence of presentation for list sets was counterbalanced across participants. The words were printed in black (RGB: 0, 0, 0), whereas the cues (Xs) were printed in green (RGB: 0, 255, 0) or red (RGB: 255, 0, 0). All stimuli (words and cues, font size 28 pt) were presented on a silver-gray background (RGB: 192, 192, 192). The participants sat approximately 80 cm from a computer screen.

In the test phase, a recognition test was conducted for the participants. Specifically, if a word had presented in the study phase (i.e., both TBR and TBF words), the participants were asked to give an "old" response, or else, give a "new" response.

Procedure

During the study phase, each trial began with a 250 ms fixation cross, followed by a 500 ms blank screen. Then, a word was presented for 500 ms. For the M-R or M-F condition, after a random blank screen of 1,300–1,700 ms, a maintenance cue (cue 1) appeared for 250 ms, followed by a 1,300–1,700 ms blank screen, then a remembering/forgetting cue (cue 2: M-R/M-F cue) appeared for 250 ms, followed by a 2,500 ms blank screen (**Figures 1A,B**); For the F condition, after a word was presented, a 1,300–1,700 ms blank screen was presented, then a forgetting cue appeared for 250 ms, followed by a 2,500 ms blank screen (**Figure 1C**).

In the test phase, each trial began with a 250 ms fixation cross, followed by a 500–800 ms blank screen, and then a word appeared for 1,500 ms. Next, a 1,000 ms blank screen was presented. The participants were asked to press "f" or "j" on the keyboard to make old/new responses to the word as quickly and accurately as possible. The key assignment for the old/new responses was counterbalanced among participants.

Data Analysis

Behavioral Data

The old response rate was defined as the percentage of old responses in each condition (i.e., hit rates to M-R, M-F, and F words and false-alarm rates to foils). A repeated-measure analysis of variance (ANOVA) with the factor word type (M-R, M-F, F, new) was performed on the old response rate.

ERP Recording and Analysis

Brain electrophysiological activity was recorded from a 64-Channel EEG recording system (Brain Products, GmbH, Germany) with references on a central midline electrode. A vertical electrooculogram (EOG) was recorded with electrodes placed below the right eye. A horizontal EOG was recorded with electrodes placed on the right canthi. All interelectrode impedance was maintained below 5 kΩ. EEG and EOG were amplified using a 0.05–100 Hz bandpass filter and continuously sampled at 500 Hz for off-line analysis.

Raw EEG data were processed offline using BrainVision Analyzer version 2.0 (Brain Products, GmbH; Gilching, Germany). For the data analysis, ERPs time-locked to the cues onset (during the study phase) were re-referenced algebraically to the average of the left and right mastoids. After ocular correction (Gratton et al., 1983), EEGs were digitally filtered with a 30 Hz low-pass filter with a 24 bit analog-to-digital converter. ERPs for all cues during the study phase were then segmented into 1,000 ms epochs surrounding stimulus presentation and baseline-corrected with respect to 200 ms pre-stimulus. Trials contaminated with EOG artifacts (mean EOG voltage exceeding ± 80 μV) or those with artifacts due to amplifier clipping, bursts of electromyographic (EMG) activity, or peak-to-peak deflection exceeding ± 100 μV were excluded from averaging. EEGs recorded in all conditions were averaged separately for each participant. The mean numbers of trials retained after artifact rejection were as follows, M-R cue: mean = 42.2, $SD = 6.7$, range, 32–52; M-F cue, mean = 42.8, $SD = 5.6$, range, 31–52; M cue: mean = 95.2, $SD = 12.8$, range, 78–119; F cue: mean = 47.4, $SD = 7.4$, range, 35–58.

For the study phase, the P2 (120–180 ms), N2/P3 (200–400 ms), and LPC (500–800 ms) time windows were chosen for statistical analysis, which corresponded to the typical latency range of the P2 (Smid et al., 1999), N2 (Folstein and Van Petten, 2008), P3 (Polich, 2007), and LPC (Patrick et al., 2015; Gao et al., 2016b) components.

The grand-averaged ERPs (**Figure 3**) showed that the maximum ERP difference during the P2 epoch distributed over the fronto-central scalp. Preliminary inspection of the data indicated that there were no ERP differences between conditions in the P2 time window at parietal electrodes (P3, Pz, P4). Therefore, data from the anterior-central recording sites [three frontal electrodes (F3, Fz, F4) and three central electrodes (C3, Cz, C4)] were selected for statistical analysis and divided into three levels (left vs. middle vs. right) of the hemisphere. Repeated-measures ANOVAs with cue type (M, M-R, M-F, F), caudality (frontal, central), and hemisphere (left, middle, right) as within-subject factors were performed on the mean amplitudes during the 120–180 ms time period.

As shown in **Figure 3**, during 200–400 ms, the M and F cues evoked a frontal N2 component, whereas the M-R and M-F cues evoked a frontal N2/P3 complex. The N2/P3a was maximally recorded at frontal sites around 300 ms (Campanella et al., 2002). Therefore, the selected epoch corresponded to the typical latency range and scalp distribution of the N2/P3 complex. Additionally, M-R/M-F cues evoked a parietal P3 component

during 200–400 ms. Therefore, data from the anterior-posterior recording sites [three frontal electrodes (F3, Fz, F4), three central electrodes (C3, Cz, C4), and three parietal electrodes (P3, Pz, P4)] were selected for statistical analysis and were factorized into three levels (left, middle, right) of the hemisphere. Repeated-measures ANOVAs with cue type (M, M-R, M-F, F), caudality (frontal, central, parietal), and hemisphere (left, middle, right) as within-subject factors were performed on the mean amplitudes during the 200–400 ms period.

During the LPC (500–800 ms) time window, data from the anterior-posterior recording sites (F3, Fz, F4, C3, Cz, C4, P3, Pz, P4) were factorized into three levels (left, middle, right) of the hemisphere. Repeated-measures ANOVAs with cue type (M, M-R, M-F, F), caudality (frontal, central, parietal), and hemisphere (left, middle, right) as within-subject factors were performed on the mean amplitudes during the 500–800 ms period.

For the ERP data, to avoid describing large amounts of statistical data concerning scalp distribution effects, only main effects or interactions that included the cue factors were reported. All effects with $>1^{\circ}$ of freedom were adjusted for sphericity violations by using the Greenhouse-Geisser correction. Main effects (or interactions) were subjected to Bonferroni-corrected pairwise comparisons (or simple effect test).

RESULTS

Behavioral Results

The old response rate was 75.5% for the M-R words, 59.3% for the M-F words, 52.1% for the F words, and 17.0% for the new words. The ANOVA results revealed a main effect of word type on the old response rate, $F_{(3, 54)} = 134.40$, $p < 0.001$, $\eta_p^2 = 0.882$. Pairwise comparisons revealed that the old response rate was higher for M-R words than for all other words, $ps < 0.001$, $ds \geq 1.41$. The old response rate was higher for M-F words relative to F words and new words, $ps \leq 0.001$, $ds \geq 0.50$; the old response rate was higher for F words than for the new words, $p < 0.001$, $d = 2.66$ (**Figure 2**).

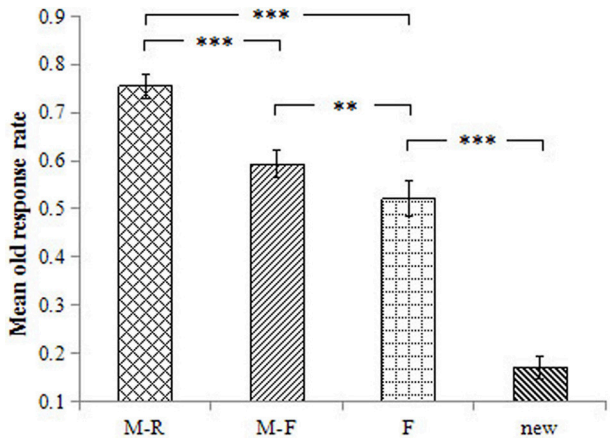

FIGURE 2 | Mean old response rate for the different conditions. Error bars represent standard error of the mean. ***$p < 0.001$, **$p < 0.01$.

Electrophysiological Results

During the P2 (120–180 ms) time window, the ANOVA results revealed a main effect of cue type, $F_{(3, 54)} = 5.295$, $p = 0.003$, $\eta_p^2 = 0.226$. Pairwise comparisons revealed that the F cue evoked smaller P2 amplitudes than the other types of cues (M-R, M-F, M), $ps \leq 0.027$, $ds \geq 0.32$. No interactions that included the cue factor were found, $ps > 0.378$.

During the 200–400 ms time window, the results revealed a main effect of cue type, $F_{(3, 54)} = 51.90$, $p < 0.001$, $\eta_p^2 = 0.742$. Both the Caudality × Cue type interaction [$F_{(6, 108)} = 13.17$, $p < 0.001$, $\eta_p^2 = 0.422$] and the Hemisphere × Cue type interaction [$F_{(6, 108)} = 22.26$, $p < 0.001$, $\eta_p^2 = 0.553$] were significant. Simple effect analyses revealed that the M-R cue evoked more positive ERPs relative to the other cues (M-F, M, F) over all scalps, $ps \leq 0.001$, $ds \geq 0.39$; the M-F cues evoked more positive ERPs relative to the M and F cues over the whole scalps, $ps \leq 0.004$, $ds \geq 0.62$; and the M cues evoked more positive ERPs relative to the F cues over all scalps, $ps \leq 0.019$, $ds \geq 0.26$.

During the LPC epoch (500–800 ms), the Cue type × Caudality × Hemisphere interaction was significant, $F_{(12, 216)} = 1.91$, $p = 0.035$, $\eta_p^2 = 0.096$. Simple effect analyses revealed that the M-R cues evoked more positive ERPs relative to M cues at central-parietal scalp electrodes (C3, Cz, C4, P3, Pz, P4), $ps \leq 0.028$, $ds \geq 0.88$; the M-F cues evoked more positive ERPs relative to M cues at central-parietal scalp electrodes (C3, Cz, C4, P3, Pz, P4), $ps \leq 0.048$, $ds \geq 0.89$; the M-F cues evoked more positive ERPs relative to F cues at C3 electrodes, $p = 0.014$, $d = 0.98$; and the M cues evoked less positive ERPs relative to the F cues at Pz and P4 electrodes, $ps \leq 0.026$, $ds \geq 0.78$.

Correlational Analyses

To better determine the functional means of observed ERP effects, Pearson correlation analysis was performed to investigate the association between the recognition accuracy (old response rate for M-R, M-F, and F words) and the amplitudes of the frontal N2 or the parietal P3 and LPC components. The amplitudes of the frontal N2 component were calculated by averaging the amplitudes from three frontal electrodes (i.e., F3, Fz, F4), as well as from three parietal electrodes (i.e., P3, Pz, P4) for the P3 and the LPC amplitudes. The results showed a positive correlation between the accuracy and the fronto-central N2 amplitudes, $r = 0.544$, $p < 0.001$. A positive correlation was found between the accuracy and the parietal P3 amplitudes, $r = 0.409$, $p = 0.002$. No correlation was found between the accuracy and the parietal LPC amplitudes, $r = 0.09$, $p = 0.505$.

DISCUSSION

The aim of the present study was to investigate whether forgetting is an active process that is more effortful than the maintenance rehearsal process. To trigger the maintenance rehearsal process, a maintenance cue was added into the item-method DF paradigm. The main findings are as follows: Consistent with previous DF studies, a typical DF effect was found. Memory performance

benefited from the maintenance rehearsal process. ERP time-locked to the cues indicated that two continuous stages were involved in the forgetting process: the attentional withdrawal process, which was reflected by decreased frontal P2 activity, and the attentional inhibition process, which was reflected by increased frontal N2 activity. The enhanced frontal ERP activity associated with the forgetting process relative to the maintenance rehearsal process suggested that forgetting is an active process.

Behavioral Results

The memory performance was superior for the studied words (M-R, M-F, F) relative to the false-alarm rate for foils, indicating that all the studied words were reasonably encoded during the study phase. Consistent with previous DF studies (Bjork and Woodward, 1973; Basden et al., 1993), a typical DF effect was observed, with higher recognition performance for M-R words relative to M-F words, indicating an enhanced encoding process for M-R words during the study phase. This DF effect demonstrated that participants had successfully manipulated the words according to different (remembering, forgetting) instructions.

M-F words showed better memory performance relative to F words. In the M-F trials, the M cue did not indicate whether the words were TBR or not. Hence, the participants would continue to keep the words in working memory through maintenance rehearsal until the M-R/M-F (cue 2) was presented. In contrast, in the F trials, the forgetting cues were presented immediately after the words, and then the maintenance rehearsal process was reduced or terminated. Therefore, the maintenance rehearsal interval was greater for M-F trials than for F trials. Previous studies demonstrated that the encoding of item information benefited from maintenance rehearsal (Woodward et al., 1973; Hockley et al., 2016). Therefore, in this study, the better recognition performance for M-F words than that for F words might have benefited from the greater maintenance rehearsal interval. Most important, M cues might successfully trigger a maintenance rehearsal process.

ERP Results

The ERP technique is most commonly used in studies of memory (see the review by Rugg and Wilding, 2000), and the P3 and LPC components are always associated with memory manipulation (Polich, 2007; Gao et al., 2016b). The ERP technique is also particularly useful in investigating the neural activity associated with cognitive control. The N2 component, which is widely generated in the medial and lateral prefrontal cortex, is always associated with the cognitive control process (see the review by Folstein and Van Petten, 2008). With the advantage of time resolution, the ERP result could reveal the time course of the neural difference between different cues.

Cognitive Control Process Triggered by Forgetting Cues: Frontal N2 Activity

Consistent with previous DF studies (Patrick et al., 2015; Gao et al., 2016a,b), the ERPs were more negative for the forgetting cue (i.e., M-F cue) relative to the remembering cue (i.e., M-R cue) during the 200–400 ms epoch. Specifically, the M-F cues evoked a frontal N2/P3 complex, which was absent for the M-R cues (i.e., a P3 component was evoked for M-R cues; see **Figure 3**). In addition, the F cue evoked a more negative N2 component relative to the M cue. Overall, forgetting cues were associated with enhanced N2 activity over the fronto-central scalp. This fronto-centrally distributed N2 component is usually observed on various measures of cognitive control, for example, the NoGo N2 (Bokura et al., 2001; Falkenstein, 2006) and the stop signal N2 (Kok et al., 2004). The N2 is often interpreted as reflecting cognitive control of attention, and N2 amplitudes were increased with attentional control improvement (Folstein and Van Petten, 2008; Espinet et al., 2012; Qi et al., 2017). Hourihan and Taylor (2006) argued that analogous to the process of preventing the implementation of an overt response, DF may involve a cognitive control process during overt encoding. The participants were encouraged to commit words to memory, and the forgetting instruction served to countermand this default covert action. In this study, the forgetting cues induced a similar fronto-central N2 activity as in motor stopping paradigms. This result might reflect a top-down cognitive control process in preventing TBF items from being further rehearsed.

The positive correlation between the fronto-central N2 amplitudes and the recognition accuracy revealed that enhanced fronto-central N2 activity was associated with decreased memory performance. It further confirmed the view that the N2 reflects the memory inhibition process. In this study, the forgetting cues (i.e., M-F and F cues) might trigger a more intensive cognitive control process relative to the remembering cues (i.e., M-R cues). Consequently, more amounts of cognitive resources would be allocated in further processing of M-R words relative to M-F and F words. This speculation was supported by the cue effect during the P3 time window.

Remembering Cue-Induced Elaborate Rehearsal Process: Parietal P3 Activity

During the P3 epoch, the mean amplitudes were increased for the remembering cues (i.e., M-R cues) relative to the forgetting cues (i.e., M-F/F cues), indicating that TBR words received a more intensive rehearsal process. The observation of a significant positive correlation between recognition accuracy and the parietal P3 amplitudes indicates that the increased P3 amplitudes might be associated with an enhanced rehearsal process. This is in line with the findings of previous DF studies, which showed that enhanced central-parietal P3 activity was positively correlated with higher subsequent memory performance (Patrick et al., 2015; Gao et al., 2016a,b). The P3 component might be a neural mark of the rehearsal process.

During the 200–400 ms time window, the M cues evoked a frontal N2 component, whereas this component was absent for the M-R cues. Instead, a P3 component was evoked (**Figure 3**). In addition, the M-R cues evoked enhanced central-parietal P3 and LPC components relative to the M cues. These results might indicate that the M cues triggered an enhanced frontal control process, and more amounts of cognitive resources were recruited for the elaborate rehearsal of M-R words. When the M cues were presented, the participants continued to keep the words in working memory through maintenance rehearsal, and these

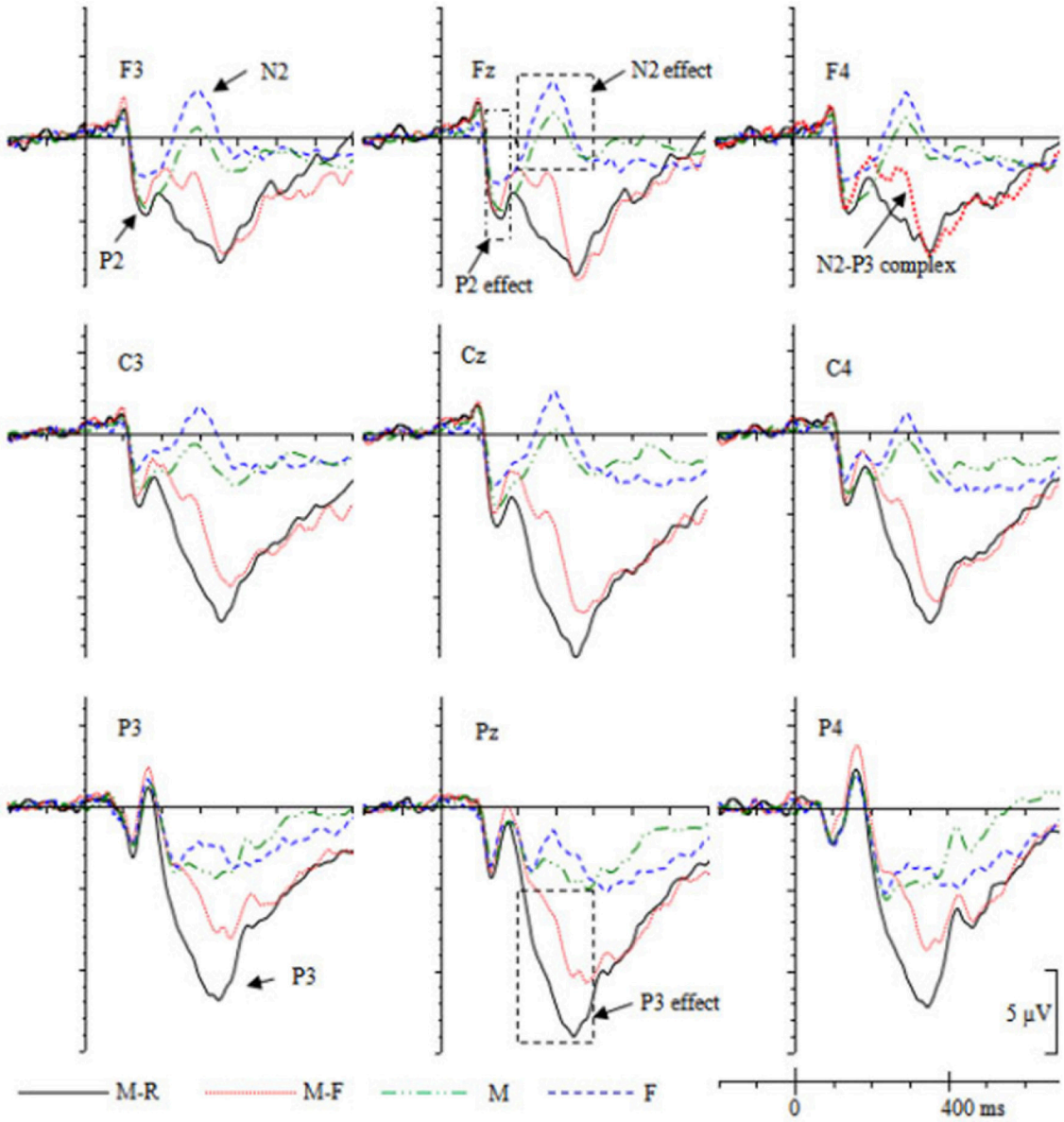

FIGURE 3 | Grand averaged ERPs ($n = 19$) for different cues during the study phase.

words did not receive elaborate rehearsal until the M-R cues were presented.

Forgetting Process vs. Maintenance Rehearsal Process

Enhanced frontal N2 and decreased central-parietal P3 and LPC activities were also found for the M cue relative to the M-F cues, indicating that a more intensive cognitive control process might be triggered by the M cues (enhanced N2), and fewer amount of cognitive resources were recruited for further rehearsal (decreased P3 and LPC). Lee and Lee (2011) found that the memory performance was improved with increased forgetting cue duration (i.e., 1 vs. 5 s). The TBF items still received processing after the cues were presented. Consistent with this view, the enhanced P3 and LPC activities associated with the M-F cue might be due to this automatic rehearsal process, which was more intensive than the maintenance rehearsal process. Similarly, this ERP effect was also observed between the F cue and the M-F cue, indicating that M-F cues received enhanced rehearsal process.

However, because the M and F cues always preceded the M-R and M-F cues, the ERP cue effect might be modulated by this cue order. The more positive-going amplitude in the M-R/M-F condition might be due to the more negative slow waves in the baseline period (−200 to 0 ms) compared with the M condition. Maintenance of information in working memory has previously

been related to negative slow waves (for a review, see Ruchkin et al., 2003; Drew et al., 2006). During the baseline time window, it is consequently possible that the ERPs are more negative for the M-R cues relative to the M cues. This potential difference in ERP amplitudes in the baseline period makes it difficult to compare the M-R and M conditions with each other, and could potentially explain the more positive-going ERPs in the M-R condition. Therefore, the ERP difference between M-F and M cues is not a good indicator to reflect the neural differences between the maintenance rehearsal and forgetting processes.

Because both the F cue and the M cue were the first cues after the words were presented, the ERP effect (F cue vs. M cue) was not influenced by the cue order effect. The F cues triggered a forgetting process and the M cues triggered a maintenance rehearsal process. Compared with the M cues, the F cues evoked a decreased fronto-central P2 component. This frontal P2 effect was also found for the F cues relative to the M-R or M-F cues. This fronto-central P2 effect reflects the enhanced selective attention process triggered by the task-relevant stimulus (Smid et al., 1999; Bergström et al., 2007; Mecklinger et al., 2009). Some DF studies also found that remembering cues evoked an enhanced frontal P2 component than forgetting cues (Cheng et al., 2012; Gao et al., 2016a). Consistent with these findings, our study suggests that increased attentional resources might be allocated to the M cues vs. the F cues.

The F cue evoked a more negative fronto-central N2 component relative to the M cues, suggesting that the F cues triggered an enhanced control process than the M cues. In addition, the ERPs were more positive for the M cues relative to the F cues over the parietal scalp during the 200–400 ms time window. As shown in **Figure 3**, at P3 and Pz electrodes, the F cues evoked an N2 component, whereas the M cues evoked a P3 component. This might indicate that the words continued to receive a rehearsal process when the M cue was presented. However, the rehearsal process was stopped or reduced when the F cue was presented. The enhanced frontal activity might provide evidence for the attentional inhibition account, which suggests that forgetting might involve an active attentional/cognitive control process.

Some researchers found that decreased P2 and enhanced N2 components were evoked for forgetting vs. remembering cues (Gao et al., 2016a). They demonstrated that DF involves two stages. During the first stage, the attentional withdrawal process stops the TBF items from being further processed; during the second stage, the memory representations of TBF items were inhibited. Similarly, in this study, decreased P2 and enhanced N2 amplitudes were evoked for the forgetting cue (i.e., F cue) relative to the M cue. This demonstrated that the forgetting cue-induced frontal activities (P2, N2) were not modulated by the remembering cue-induced elaborate rehearsal process. It further supported the view that forgetting is an active process that involves attentional withdrawal (P2 epoch) and cognitive control (N2 epoch).

The M cues evoked reduced LPC amplitudes relative to the F cues at parietal electrodes (i.e., Pz, P4), suggesting that the TBF words received enhanced, further processing after the F cues were presented. One possibility is that it might reflect enhanced working memory maintenance and/or rehearsal process for the F condition relative to the M condition. Lee (2012) suggested that TBF words were automatically processed to the extent that cognitive resources remained available. In addition, some studies have found that the forgetting cue in an item-method DF paradigm might prompt subjects to process the TBF items (Zwissler et al., 2015; Gao et al., 2016b). Therefore, the ERP cue effect during the LPC epoch might reflect that TBF words were automatically rehearsed, and more amounts of cognitive resources were recruited for the F cues relative to the M cues. However, no significant correlation was found between the recognition performance and LPC amplitudes. An alternative explanation for this LPC effect might be that, in order to remember as many TBR words as possible, the participants might cumulatively rehearse the TBR words from preceding trials when the forgetting cue was presented (Fawcett and Taylor, 2008, 2012). This LPC activity might be associated with the study phase retrieval or cumulative rehearsal process.

Zwissler et al. (2015) suggested that frontal brain activation associated with forgetting cues might result from either non-inhibitory processes, such as attention orienting, conflict monitoring, or unsuccessful inhibition attempts. The frontal activity (i.e., P2, N2 activity) found in this study might reflect the attention orienting and cognitive control processes. MacLeod (2007) defined cognitive inhibition as "the stopping or overriding of a mental process, in whole or in part, with or without intention." In consideration of this definition, the present findings might reveal the role of inhibition in DF as attempting to suppress the ongoing encoding, although the TBF words were automatically processed.

There was a limitation to this paradigm. There were twice as many M cues as F cues in this study. In other words, for the participants, after the words were presented, the F cues showed a lower probability of occurrence relative to the M cues. Therefore, the magnitude of the cue effect (F cues vs. M cues) during the N2 epoch might be enhanced owing to this oddball effect, although the maximum oddball N2 difference distributed over the posterior rather than the anterior scalp (see the review by Folstein and Van Petten, 2008). Additionally, previous ERP studies employing the oddball paradigm demonstrated that rare stimuli evoked enhanced P3 and LPC activity relative to frequent stimuli (Campanella et al., 2002; Denecke et al., 2004). Therefore, the cue effect between F and M cues during the P3 and LPC time windows might also be affected by the oddball effect. Future studies could adjust the proportion of different trials to eliminate this oddball effect.

CONCLUSIONS

This study aimed to compare the neural activity of maintenance rehearsal vs. DF. Compared with the M

cue, the F cue evoked a decreased frontal P2 component and an enhanced frontal N2 component, indicating that DF is an active process that involves a frontal control process. In addition, DF might be more effortful relative to maintenance rehearsal. Furthermore, the cognitive control process might play an important role in intentional forgetting.

AUTHOR CONTRIBUTIONS

HG and MQ: designed the experiment. HG: conducted the experiment and analyzed the data. HG and MQ: wrote the manuscript. All authors edited and revised manuscript and approved final version of manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grants 30970888).

ACKNOWLEDGMENTS

We would like to thank Jiazhan Wang for his help in collecting data.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Neuroscience

ORIGINAL RESEARCH
published: 28 March 2018
doi: 10.3389/fnins.2018.00204

No Gender Differences in Egocentric and Allocentric Environmental Transformation After Compensating for Male Advantage by Manipulating Familiarity

Raffaella Nori[1], Laura Piccardi[2,3], Andrea Maialetti[4], Mirco Goro[4], Andrea Rossetti[4], Ornella Argento[4] and Cecilia Guariglia[3,4]*

[1] Department of Psychology, University of Bologna, Bologna, Italy, [2] Life, Health and Environmental Science Department L'Aquila University, L'Aquila, Italy, [3] Neuropsychology Unit, IRCCS Santa Lucia Foundation, Rome, Italy, [4] Department of Psychology, University of Rome, Rome, Italy

OPEN ACCESS

Edited by:
Alfredo Meneses,
Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Mexico

Reviewed by:
Zoltan Nadasdy,
NeuroTexas Institute and Research Foundation, United States
Aaron Wilber,
Florida State University, United States

***Correspondence:**
Raffaella Nori
raffaella.nori@unibo.it

Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: 01 August 2017
Accepted: 14 March 2018
Published: 28 March 2018

Citation:
Nori R, Piccardi L, Maialetti A, Goro M, Rossetti A, Argento O and Guariglia C (2018) No Gender Differences in Egocentric and Allocentric Environmental Transformation After Compensating for Male Advantage by Manipulating Familiarity. Front. Neurosci. 12:204. doi: 10.3389/fnins.2018.00204

The present study has two-fold aims: to investigate whether gender differences persist even when more time is given to acquire spatial information; to assess the gender effect when the retrieval phase requires recalling the pathway from the same or a different reference perspective (egocentric or allocentric). Specifically, we analyse the performance of men and women while learning a path from a map or by observing an experimenter in a real environment. We then asked them to reproduce the learned path using the same reference system (map learning vs. map retrieval or real environment learning vs. real environment retrieval) or using a different reference system (map learning vs. real environment retrieval or vice versa). The results showed that gender differences were not present in the retrieval phase when women have the necessary time to acquire spatial information. Moreover, using the egocentric coordinates (both in the learning and retrieval phase) proved easier than the other conditions, whereas learning through allocentric coordinates and then retrieving the environmental information using egocentric coordinates proved to be the most difficult. Results showed that by manipulating familiarity, gender differences disappear, or are attenuated in all conditions.

Keywords: gender differences, allocentric frames of reference, egocentric frames of reference, change of perspective, learning time

INTRODUCTION

Human beings orient themselves through the environment by using different strategies to represent the space. The notion of "frame of reference" refers to the way in which individuals represent landmarks and their spatial location as well as their own position with respect to the environmental objects.

In spatial cognition, individuals may use two different spatial frames of references: "egocentric" (body-centered) and "allocentric" (world-centered) (Burgess, 2006, 2008; Arleo and Rondi-Reig, 2007). Specifically, an individual may locate environmental features by (a) referring to his own position, namely an egocentric frame of reference or (b) referring to the spatial and configurational

properties of environmental features, namely an allocentric frame of reference (Galati et al., 2000; for a review see Avraamides and Kelly, 2008).

For example, when individuals actually navigate through an environment they memorize spatial locations considering them with respect to their position, whereas when they plan or study a path by observing it on a map they represent the space regardless of their own position. Both humans and animals form an environmental representation by integrating internal cues (e.g., self-motion; proprioceptive information; visual cues) with external ones (e.g., the relationships between environmental landmarks; positional and directional environmental cues: Cheng, 1986; Jacobs and Schenk, 2003). Therefore, the "map-like" environmental representation depends on egocentric and allocentric reference frames and the continuous translation of information coming from these two systems.

According to Wolbers and Hegarty (2010), spatial navigation requires three essential elements: (1) Spatial cues, (2) Computational mechanisms, and (3) Spatial representations. Spatial cues have a role in extracting information about our own position in the environment. They might refer to either environmental cues (i.e., such as landmarks, geometric structure of the environment) or self-motion cues (i.e., such as proprioceptive, vestibular, and motion cues). Computational mechanisms include both spatial computations and more general executive processes. On the one hand, spatial computations include space perception, translating between egocentric and allocentric frames of reference, computing distance and directions toward an invisible goal, imagining shifts in spatial perspective. On the other hand, executive processes involve novelty detection, selection, and maintenance of the navigational goal, route planning, and conflict resolution. Spatial representations include both offline and online spatial representations. Most of the models of human spatial navigation focused on the attributes of the spatial representations, that is the way in which spatial knowledge is organized.

As pointed out by Mou et al. (2006), a path requires the computation of a precise self-to-object spatial relation to guide locomotion, while planning a route to a distant goal and maintaining a sense of direction in a large-scale environment requires object-to-object spatial relation. The egocentric frame of reference considers the body as the center of the environmental organization so the spatial mental representation is biased by the relation between the body's position and the spatial location. On the contrary, the allocentric frame of reference is specified independently of the body's position (e.g., Waller et al., 2002). As a consequence, during navigation, individuals process egocentric and allocentric environmental cues that have a role in building and retrieving topographic long-term memory. Contemporary models of human spatial memory and navigation attempt to specify the role of both egocentric and allocentric information. Wang and Spelke, (2000, 2002) suggested that spatial memory was solely supported by egocentric references. Specifically, they suggested the existence of two stages, namely an egocentric process and a geometric module. The former involves a viewpoint-dependent scene recognition and a spatial updating of location by self-motion information whereas the latter has no role in representing locations but only in representing the geometry of the environment in spite of the individual's position. From this point of view, an allocentric frame of reference is not openly considered, if not with the purpose to support reorientation when the path integration system (i.e., the capacity to use cues generated by one's own movements to update one's position in the environment) breaks down. However, experimental data indicate a "two-system" model of parallel egocentric and allocentric representations in memory (e.g., Burgess et al., 2001; Burgess, 2006; Waller and Hodgson, 2006). Mou et al. (2004) proposed another model of spatial memory and navigation including an egocentric and an environmental subsystem. The former computes and represents the transient self-to-object spatial relations needed for locomotion, and decays rapidly in the absence of perceptual input or rehearsal. The latter, instead, is responsible for representing the characteristics of a familiar environment in an orientation-dependent manner. Both subsystems are based on an intrinsic reference system (e.g., Shelton and McNamara, 2001).

Researchers taking into account the role played by the reference systems in several actions hypothesize that they may form specialized cognitive mechanisms that rely on specific neural networks. In particular, it appears that the hippocampus and the medial temporal lobe provide allocentric environmental representations, while the parietal lobe provides egocentric representations, and the retrosplenial cortex and parieto-occipital sulcus allow both types of representation to interact (for a review see, Burgess, 2008).

From a neural point of view, several studies have shown that egocentric navigation is sub-served by a set of areas related to landmark knowledge (i.e., the parahippocampal place area, Epstein and Ward, 2010), egocentric spatial representation by the parietal cortex (i.e., precuneus and cuneus, inferior parietal lobe) and heading information by the retrosplenial cortex. Instead, allocentric navigation seems mainly related to the hippocampal cortex (Tolman, 1948; O'Keefe and Nadel, 1978; Maguire et al., 1998). Moreover, fMRI studies have shown activations in the hippocampal formation, parietal cortex and retrosplenial regions during tasks involving both egocentric (Galati et al., 2000; Wolber et al., 2004; Latini-Corazzini et al., 2010) and allocentric (Iaria et al., 2007; Latini-Corazzini et al., 2010) frames of reference.

In addition, an ALE meta-analysis by Boccia et al. (2014) demonstrated that the allocentric and egocentric frames of reference were subtended by the same areas, but the latter elicits greater activation in the right precuneus, middle occipital lobe, and angular gyrus.

Studies that investigate the use of egocentric and allocentric frames of reference assume that they are determined mainly by innate factors such as gender (e.g., Chai and Jacobs, 2010), age (e.g., Moffat and Resnick, 2002), or familiarity (e.g., Nori and Piccardi, 2011, 2012). Other studies have considered that the egocentric and allocentric frames of reference may also be affected by external factors such as stimulus salience (e.g., Wolbers and Hegarty, 2010), the availability of landmarks or the experience with these stimuli (Wolbers and Hegarty, 2010). Furthermore, the type of navigational tasks elicits the use of one system rather than the other. For example, route learning tasks

elicit egocentric coordinates (e.g., Nemmi et al., 2013), while place learning tasks elicit allocentric ones (e.g., Woolley et al., 2010). However, some studies have revealed that people are able to switch between the two different frames of reference (e.g., Iaria et al., 2003; Etchamendy and Bohbot, 2007; Iglói et al., 2009). Recently, Livingstone-Lee et al. (2014) have studied the effects of performing allocentric and egocentric training in men and women for a subsequent spatial task in which the use of both allocentric and egocentric frames of reference were equally efficient. The results showed no evidence of gender differences in the use of egocentric/allocentric coordinates and in navigation performance, suggesting that individuals may be trained to use one system rather than another. Piccardi et al. (2011a) showed that gender differences in the use of egocentric and allocentric references emerge only in adverse learning conditions when the task requires high spatial skills.

Altogether these studies raise the important issue of needing to deepen our knowledge on what factors might influence the use of ego/allocentric coordinates.

There is also evidence that memories for pathways are viewpoint dependent. As spatial information is encoded according to an orientation-dependent view, the original learning perspective constitutes the primary frame of reference irrespective of the use of egocentric or allocentric coordinates (e.g., Presson and Montello, 1994; Sholl and Nolin, 1997; Shelton and McNamara, 2001). As Piccardi et al. (2011a) pointed out, there is little agreement about which factors are important in attenuating or eliminating orientation-dependent path representations: some studies highlight the environmental characteristics of the path that people have to acquire and remember (e.g., Sholl and Nolin, 1997) whereas others focus on the strategy used for acquiring spatial information (e.g., Rossano et al., 1995; Nori et al., 2006). Piccardi et al. (2011a) observed that when people have the possibility to learn a path without a time limit, basing their strategies on egocentric coordinates, both men and women are good at performing directional judgments irrespective of the learning orientation. In this work, the authors investigated the ability of 106 (55 males, 51 females) college students to recall an 8-step path from different viewpoints after moving directly on the path or by studying the same layout printed on a map. Participants did not have any time limit during the Learning phase. For each participant, authors computed the time and the number of repetitions necessary to learn the path.

Results showed that by allowing longer duration of familiarization and more practice repetitions for females than males markedly reduced the gender difference (for details see Piccardi et al., 2011a). For such a reason, in the present study we have set a time limit corresponding to 3 min and 1 s for men and 3 min and 30 s for women and a number of repetitions corresponding to three times for men and four times for women, adopting the same means and standard deviations obtained by participants in Piccardi et al. (2011a).

This is true also during navigation in virtual environments (Nori et al., 2015a,b).

The aim of the present study was to investigate the presence of gender differences in learning a pathway in different frames of reference (ego/allocentric) and in translating from one frame of reference to another, considering the different time of familiarization for men and women. We also hypothesize that women perform better and more quickly in egocentric as opposed to allocentric frames of reference. Specifically, we hypothesized that adopting the same frame of reference assumed during the learning phase also in the retrieval phase may make participants more accurate and faster in recalling the previously learnt pathway. Vice versa, translating spatial information learnt in one frame of reference to another may affect the accuracy and speediness in the recalling of the learnt path. With respect to gender, we also expected that the translating computation from one frame of reference to another would be more difficult for women than men. A secondary aim of this work was to analyze if increasing the familiarity with the experimental setting (acting through the exposure time to the map or the number of repetitions of the path in the real environment) may produce an improvement in the performance making it easier to translate from one frame of reference to another.

METHOD

Participants

The study involved 160 College students (83 women), recruited in the Sapienza University of Rome, aged between 18 and 30 years ($M = 24.91$ years $S.D. = 2.41$ years; Men, $M = 25.76$ years, $S.D. = 2.20$ years; Women, $M = 24.03$ years, $S.D. = 2.30$ years). Thirteen participants were left-handed and eight ambidextrous (Salmaso and Longoni, 1983). Participants were randomly assigned to one out of four experimental conditions, namely: egocentric frame of reference condition (EC), 40 participants (20 women); allocentric frame of reference condition (AC), 41 participants (21 women); egocentric/allocentric frames of reference condition (EAC), 42 participants (22 women) and allocentric/egocentric frames of reference condition (AEC), 37 participants (20 women). This study was carried out in agreement with the Declaration of Helsinki with written informed consent from all subjects. The protocol was approved by the Local Ethical Committee of the Sapienza University.

APPARATUS AND PROCEDURE

Condition 1. EC

Egocentric Learning Phase

Each participant was tested individually. We used an enlarged version of the WalCT (Piccardi et al., 2008, 2013), that is the M-WalCT (used in Piccardi et al., 2011b, 2014, 2016; Nori et al., 2015a,b) in which 18 squares (3 × 3 cm) are placed on a carpet (5 × 6 m) in a scattered array (**Figure 1A**). To induce route acquisition, the four cardinal points (i.e., North, South, East, West) are indicated outside the carpet. The walls are completely covered with curtains that hide all external landmarks (i.e., doors, heaters, etc.). In this learning condition, participants had to learn four different 8-step sequences. The experimenter demonstrated each sequence by walking on the carpet and stopping on each square for 2 s. On the basis of the findings of Piccardi et al. (2008, 2011a,b), which showed that women require more time to acquire a path than men, and in accordance with Piccardi et al. (2011b,

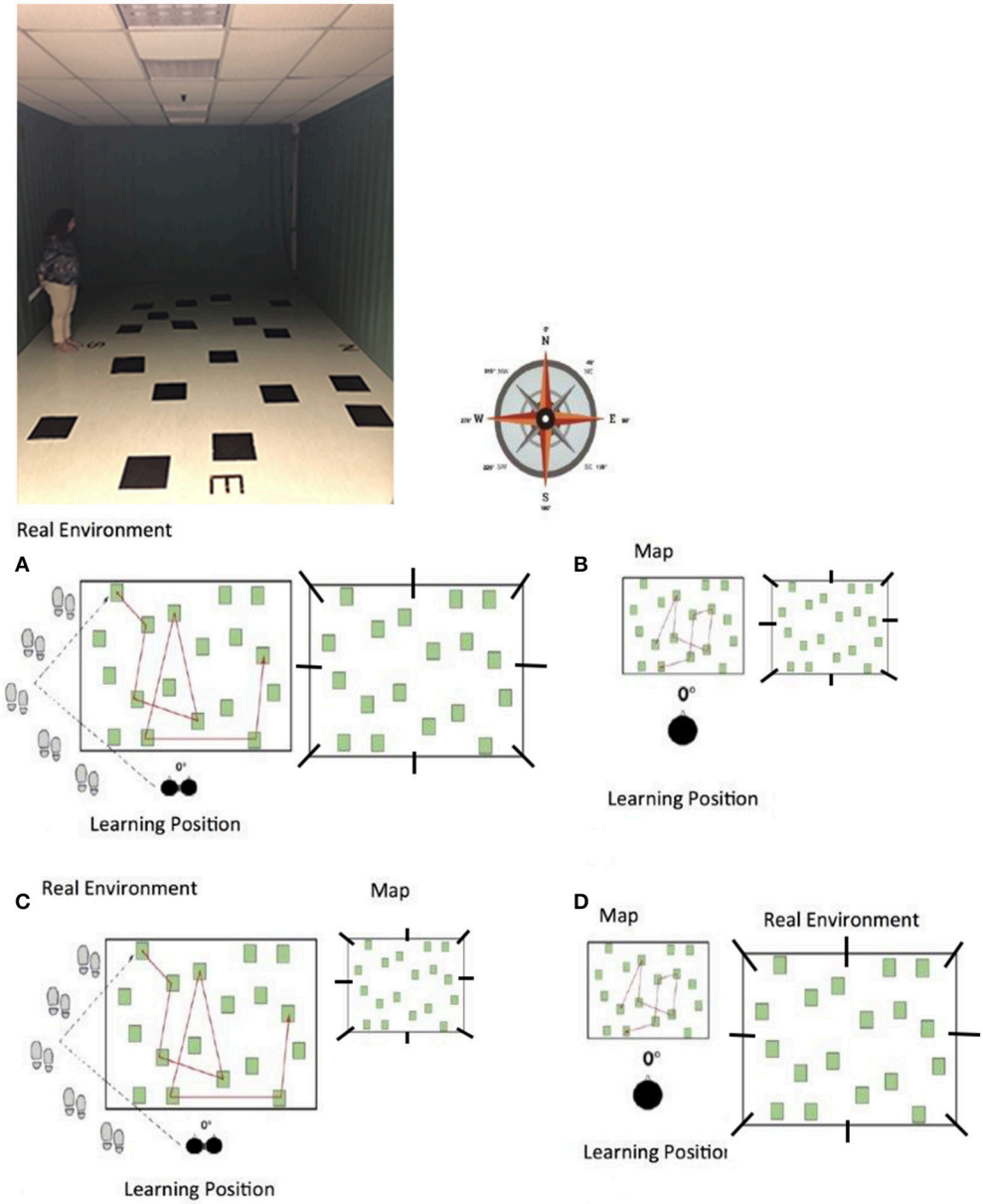

FIGURE 1 | The photo of experimental apparatus and the different experimental condition with the different angle degree where to reproduce the path: EC **(A)** egocentric frame of reference with the same reference system both for learning and retrieval; AC **(B)** allocentric frames of reference with the same reference system both for learning and retrieval; EAC **(C)** different reference system condition, that is to acquire spatial information using an egocentric frame of reference and to retrieve it using allocentric coordinates; AEC **(D)** different reference system condition, that is to acquire spatial information using an allocentric frame of reference and to retrieve it using egocentric coordinates.

2014), we considered different times of learning for men and women: the experimenter demonstrated each path three times for men and four times for women. Blindfolded participants were seated in a wheelchair located at the end of the room and were then wheeled, unblindfolded, toward the path. Each participant was then asked to stand up and was taken to the beginning of the path where s/he was led by the experimenter along eight different squares. At the end of each path, the participant was re-seated in the wheelchair and wheeled in a random and meandering route back to the initial location for his/her next walk along the same path, until it had been followed three times for men and four for women. Each walk took ∼40 s. Each participant learned four paths and was tested in eight different angle degrees for each path for a total of 32 trails.

Egocentric Testing Phase

After the learning phase, the participants were pushed in the wheelchair again and, unblindfolded, placed in front of the path

and were asked to reproduce the path they had learned before in the same or in different perspectives (0°–45°–90°–135°–180°–225°–270°–315°) by walking on the layout. Specifically, the participant was placed in front of the layout in the which s/he had to reproduce the path. The order of the different degrees of reproduction was determined randomly for each path and then the same order was used for all participants (Nori et al., 2006). For each path, the experimenter recorded the points/locations on the path correctly reproduced, that is points/locations in the exact sequence order. A hand-held stopwatch was used to record the planning time, that is the time elapsed before starting the path that had to be reproduced, and response time, that is the time people took to solve the task, including the planning time.

Condition 2. AC

Allocentric Learning Phase

Each participant was tested individually. We used a map reproduction printed on an A4 sheet of paper of the M-WalCT used in EC, reproduced in 1:10 scale and the same four different 8-step sequences that the participants had to learn (see **Figure 1B**). On the basis of Piccardi et al. (2011b, 2014), we considered different times of learning for men and women: the experimenter demonstrated each path for 3 min and 1 s for men and 3 min and 30 s for women. Blindfolded participants were seated in a wheelchair located at the end of the room. They were then wheeled, unblindfolded, to a table where they found the first of four paths printed on the map and studied it for the amount of time established for men and women respectively. At the end of the learning phase on each map, the participant was re-seated in the wheelchair and wheeled in a random and meandering route back to the table. In spite of for the allocentric condition to perform participants' disorientation is useless, we have disoriented participants for making the experimental procedure comparable to that of the egocentric condition. Each participant learned four paths printed on a sheet of paper and was tested in eight different angle degrees for each path for a total of 32 trails.

Allocentric Testing Phase

After the learning phase, the participants were asked to reproduce with a pen the path they had previously learned from the same or different perspectives (0°–45°–90°–135°–180°–225°–270°–315°) on a blank map, that is without the path printed on it. Specifically, the blank map was placed in front of the participant in the perspective from which s/he had to reproduce the path. To learn a path from a map requires an allocentric reference frame because it requires to process the whole path whereby the participant represents and updates his/her position in the environment. In such a way, generally, this kind of task is solved by using a reference system external to the body and anchored in the environment (see for example Klatzky, 1998). In such a reference system, the reference directions or axes are stable with respect to the local environment, that is the relationship among landmarks along a path without being centered on the body (Meilinger et al., 2014). We believe that learning a path from a map satisfies this constrain as a consequence we considered this task part of the allocentric testing phase.

The order of the different degrees of reproduction was determined randomly for each path and then the same order was used for all participants (Nori et al., 2006). For each path, the experimenter recorded the points/locations on the map correctly reproduced. A hand-held stopwatch was used to record the planning time, that is the time elapsed before starting the path that had to be reproduced, and response time, that is the time people took to solve the task, including the planning time.

Condition 3. EAC

Egocentric Learning Phase

The learning phase was the same as in Condition 1, EC: participants had to learn four different 8-step sequences of the M-WalCT. The experimenter demonstrated each sequence by walking on the carpet and stopping on each square for 2 s (see **Figure 1C**); the experimenter demonstrated each path three times for men and four times for women. Blindfolded participants were seated in a wheelchair located at the end of the room and were then wheeled toward the path. The participant was then asked to stand up and was led to the beginning of the path, where s/he was led by the experimenter along eight different routes. At the end of each path, the participant was re-seated in the wheelchair and wheeled in a random and meandering route back to the initial location for his/her next walk along the same path, until it had been followed three times for men or four for women. Even in this case, each participant was submitted to a total of 32 trails.

Allocentric Testing Phase

After the learning phase, as in Condition 2 AC, the participants were asked to reproduce the path they had previously learned from the same or different perspectives (0°–45°–90°–135°–180°–225°–270°–315°) on a blank map of the M-WalCT. The blank map was placed in front of the participant in the perspective from which s/he had to reproduce the path. The order of these different degrees of reproduction was determined randomly for each path and then the same order was used for all participants (Nori et al., 2006). For each path, the experimenter recorded the points/locations on the map correctly reproduced. A hand-held stopwatch was used to record the planning time, that is the time elapsed before starting the path that had to be reproduced, and response time, that is the time people took to solve the task, including the planning time.

Condition 4. AEC

Allocentric Learning Phase

The learning phase was the same as for Condition 2, AC: the experimenter demonstrated each path printed on a map for 3 min and 01 s for men and 3 min and 30 s for women (see **Figure 1D**). Successively, blindfolded participants were seated in a wheelchair located at the end of the room. They were then wheeled to the table where they found the first of four paths. At the end of the learning phase on each map, the participant was seated in the wheelchair and wheeled in a random and meandering route back

to the table. Even in this case, each participant was submitted to a total of 32 trails.

Egocentric Testing Phase

After the learning phase, as in Condition 1, EC: the participants were asked to reproduce the path they had previously learned on a map from the same or different perspectives (0°–45°–90°–135°–180°–225°–270°–315°) by walking on the real environment. Participant was placed in front of the layout in the perspective from which s/he had to reproduce the path. As for the previous conditions, the order of these different degrees of reproduction was determined randomly for each path and then the same order was used for all participants (Nori et al., 2006). For each path, the experimenter recorded the points/locations on the path correctly reproduced. A hand-held stopwatch was used to record the planning time, that is the time elapsed before starting the path that had to be reproduced, and response time, that is the time people took to solve the task, including the planning time.

RESULTS

We compared the four conditions considering a three-way analysis of variance with mixed designs with two levels of the between-variable "gender" (men/women), four levels of between-variable "condition" (EC – AC – EAC – AEC) and the 8 "degrees" of path reproduction as repeated factor (0°–45°–90°–135°–180°–225°–270°–315°). As dependent variable we considered both the mean number of locations correctly reported (accuracy; minimum score = 0, maximum score = 8) and the response time (s.) for each degree (perspective) on the four paths. Both for accuracy and response time, we handled the data, and we averaged them for each participant. In order to analyse if the assumption of homogeneity of variance is met we performed the Levene test (Levene, 1960) on accuracy [0°: $F_{(1, 158)} = 3.99$, $p = 0.52$; 45°: $F_{(1, 158)} = 0.05$, $p = 0.81$; 90°: $F_{(1, 158)} = 0.01$, $p = 0.92$; 135°: $F_{(1, 158)} = 0.19$, $p = 0.66$; 180°: $F_{(1, 158)} = 0.03$, p =.84; 225°: $F_{(1, 158)} = 0.06$, $p = 0.80$; 270°: $F_{(1, 158)} = 0.17$, $p =$ 67; 315°: $F_{(1, 158)} = 0.24$, $p = 0.62$], response time [0°: $F_{(1, 158)}$ =3.09, $p = 0.08$; 45°: $F_{(1, 158)}$ =0.1.29, $p = 0.25$; 90°: $F_{(1, 158)} = 1.40$, $p = 0.23$; 135°: $F_{(1, 158)} = 0.00$, $p = 0.93$; 180°: $F_{(1, 158)} = 1.10$, $p = 0.29$; 225°: $F_{(1, 158)} = 2.55$, $p = 0.11$; 270°: $F_{(1, 158)} = 1.48$, p =0.22; 315°: $F_{(1, 158)} = 1.78$, $p = 0.18$], and planning time [0°: $F_{(1, 158)}$ =3.20, $p = 0.07$; 45°: $F_{(1, 158)} = 0.1.51$, $p = 0.22$; 90°: $F_{(1, 158)} = 4.71$, $p = 0.03$; 135°: $F_{(1, 158)} = 1.35$, $p = 0.24$; 180°: $F_{(1, 158)} = 1.28$, $p = 0.25$; 225°: $F_{(1, 158)} = 1.20$, $p = 0.27$; 270°: $F_{(1, 158)} = 7.29$, $p = 0.00$; 315°: $F_{(1, 158)} = 7.22$, $p = 0.00$]. The results show that the assumption of homogeneity of variance is met for accuracy and response time but not for some perspectives (angles) in planning time so we did not perform any further analysis on this variable.

Number of Locations Correctly Reported (Accuracy)

The main effect of "gender" was not statistically significant [$F_{(1, 152)} = 0.66$, $p = 0.41$, $\eta^2 = 0.00$; Men M: 5.93, $S.D.$ = 0.19, Women $M = 6.16$, $S.D. = 0.18$], nor was the interaction "gender x condition" [$F_{(3, 152)} = 1.13$, $p = 0.33$, $\eta^2 = 0.02$]. The main effect of "condition" was statistically significant [$F_{(3, 152)} = 10.77$, $p < 0.001$, $\eta^2 = 0.17$]. *Post-hoc* Bonferroni revealed that the EC is easier than all the others ($p_s < 0.01$: EC, M = 7.25 $S.D.$ = 0.27; AC, M = 5.85 $S.D.$ = 0.26; EAC, M = 6.03 $S.D.$ = 0.26; AEC, M = 5.05 $S.D.$ = 0.28). The main effect of "degrees" was statistically significant [$F_{(7, 152)} = 2.17$, $p =$ 0.01, $\eta^2 = 0.01$]. *Post-hoc* Bonferroni revealed that it is easier to remember a path from 315° (M = 6.18, $S.D.$ = 0.37) than from 90° (M = 5.97, $S.D.$ = 0.14) and 225° (M = 5.99, $S.D.$ =.14). No other significant results were found. The interaction "condition x degrees" was statistically significant [$F_{(21, 152)} =$ 2.84, $p < 0.001$, $\eta^2 = 0.17$]. Specifically, *post-hoc* Bonferroni revealed that remembering a path in a real congruent condition (EC) is easier than in a map/real incongruent condition (AEC) for all perspectives ($p_s < 0.001$). Moreover, remembering a path in a real congruent condition (EC) is easier than in a map congruent condition (AC) from 135° and 225° perspectives (see **Figure 2**). No other significant differences were revealed.

Response Time

The main effect of "gender" was statistically significant [$F_{(1, 152)}$ = 4.25, $p < 0.05$, $\eta^2 = 0.02$], men are faster than women (men, M = 19.96s., $S.D.$ = 0.1.30s.; women, M = 22.70s., $S.D.$= 0.81.25s.). Moreover, the main effect of "condition" was also statistically significant [$F_{(3, 152)} = 13.68$, $p < 0.001$, $\eta^2 = 0.21$]. *Post-hoc* Bonferroni showed that remembering a path in AC (M = 27.90s., $S.D.$ = 1.78s.) was slower than in AEC (M = 11.97s, $S.D.$ = 1.88s.) and EC (M = 19.63s., $S.D.$ = 1.80s.). Moreover, the AEC is faster than EC and EAC (M = 23.82s., $S.D.$ = 1.76s.). The main effect of "degrees" was statistically significant [$F_{(7, 152)} = 18.71$, $p < 0.001$, $\eta^2 = 0.11$]: recalling a path from 225°-270°-315° is slower than all other degrees ($p < 0.05$).

The interaction "condition x degrees" was also statistically significant [$F_{(21, 152)} = 8.80$, $p < 0.001$, $\eta^2 = 0.14$]. Specifically, *post-hoc* Bonferroni showed that remembering a path in the AEC is faster than in AC at 0°–90°–135°–180° ($p_s < 0.01$); remembering a path in AC is slower than in EC and EAC at 225° ($p_s < 0.05$) while it is faster than in EC and in EAC at 0°–135°–180° ($p_s < 0.05$); remembering a path in AEC is faster than in EAC at 270° ($p < 0.05$). Descriptives are shown in **Figure 2**. No other significant differences are shown.

DISCUSSION

To our knowledge, the present study was the first to investigate the presence of gender differences in retrieving environmental information learnt in the same or different frames of reference. In particular, our intent was to analyse whether the performance of men and women in retrieving a path from different points of view using the same reference both in the learning and retrieval phases is easier and faster than one in which different references are used. We also wanted to investigate whether gender differences could be reduced by increasing the familiarity with the experimental setting by manipulating the time of map exposure or the number of repetitions to learn a pathway.

Our results partially confirm our hypothesis. Gender differences in remembering a path correctly were absent in

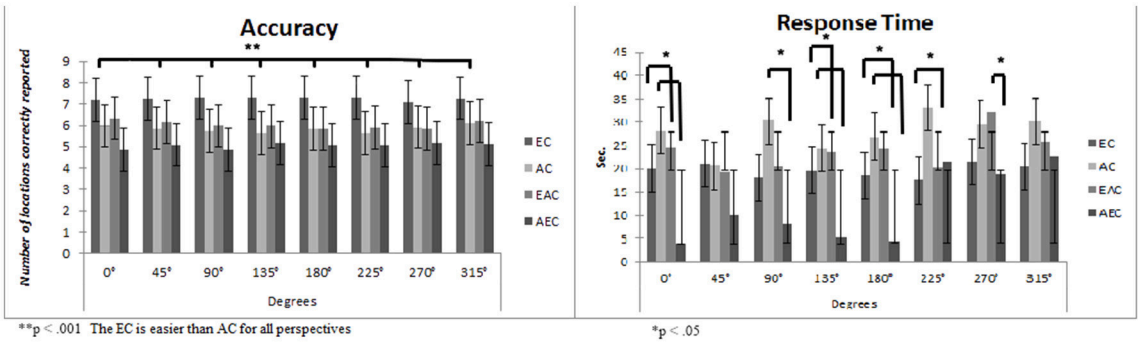

FIGURE 2 | Means and standard deviations considering the four Experimental Conditions (EC, Egocentric Condition; AC, Allocentric Condition; EAC, Egocentric Allocentric Condition; AEC, Allocentric Egocentric Condition) and the eight Degrees in terms of Accuracy and Response Time. The panel on the **right** represents participants' performances in terms of Accuracy, on the y axes is reported the number of location correctly reported (maximum score = 8), while in x axes are reported the degree of reproduction. The panel on the **left** represents participants' performances in terms of Response Time. Specifically, on the y axes is reported the time spent in reproducing the path expressed in seconds, while on the y axes is reported the different degree of path starting.

both egocentric and allocentric reference frames. In particular, we found that by manipulating familiarity, gender differences disappeared or attenuated. This result differs from that of several studies in which men outperform women in perspective-taking tasks (e.g., for a review see Coluccia and Louse, 2004). However, in these studies men and women were exposed to the same short time to acquire spatial information. Already, Piccardi et al. (2011a,b) found that computing the individual learning time allows gender differences in perspective taking tasks to be eliminated. Therefore, also computational spatial operations like the transformation of spatial information from egocentric to allocentric frames of reference and vice versa do not elicit a gender effect. This supports the evidence that when spatial information is stored and consolidated, all individuals are able to process and re-elaborate it. This result is in line with studies which demonstrated that by increasing the familiarity with the environment even poor spatial navigators were able to perform very complex tasks: the higher the individuals' familiarity with the environment, the better their performances (Iachini et al., 2009; Nori and Piccardi, 2011; Piccardi et al., 2011a). Indeed, as pointed out by Montello (1998) additional locomotor and perceptual experience of the environment, as well as familiarity with the place, result in more extensive, complete, and accurate knowledge. So the two concepts are related and specifically, increasing learning time increases familiarity with the environment, thus allowing a more complete knowledge of the experimental environment. Our results are also in line with Iachini et al. (2009), who investigated how familiarity and gender have an effect even in the frames of reference used in memory to represent a real environment. Their results showed that males were more accurate and faster than females in detecting environmental changes, in particular when participants were unfamiliar with the environment. Considering that in our experiment, the degree of familiarity with the environment was measured in terms of the time necessary to learn the experimental array, we found that when time and repetitions are sufficient, women are as efficient as men. However, it is interesting that gender differences are still present in solving the task: men are quicker than women. Therefore, familiarity is important to eliminate gender differences in terms of accuracy but it is not sufficient to eliminate gender differences in response time. This result could be explained by considering the model of Coluccia and Louse (2004), who proposed that gender differences emerged according to the cognitive demand of the spatial tasks, which could be attributed to the visuo-spatial working memory load. Specifically, gender differences emerged only when tasks required a high integration and transformation of visually imagined material: given the necessary time to acquire spatial information females may rotate the path accurately even if they take longer than males to do so, since they are accurate but still slow in performing the mental rotation of the environment.

Another result that emerged from our study is that all participants showed the same level of accuracy in all perspectives they assumed to recall the path. In general, all preferred clockwise and right body axes starting positions with respect to counter clockwise and left body axes positions, probably because the participants were right-handed (see also: Sholl and Nolin, 1997). The time of response is predicted by the time employed to mentally process the cognitive map of the environment. In particular, we found that the planning time predicted some perspectives in terms of time of performance.

As far as the effect of learning and retrieval spatial information is concerned, using the same reference frame, we found that the egocentric frame of reference condition (EC) is the easiest for all participants in recalling a path from different perspectives, while the allocentric frame of reference condition (AC) resulted in a worse performance. This is in line with Wang and Spelke's model (Wang and Spelke, 2000, 2002), which suggests that the egocentric coordinates are the preferred system for both men and women, even if the use of allocentric coordinates could be not completely excluded, and it is possible that participants may simultaneously use an allocentric reference in which they themselves are the references for the spatial information (Burgess, 2006). Very likely, men are more proficient than

women in adverse learning conditions (e.g., temporal pressure or when the task requires a high cognitive load) (Coluccia and Louse, 2004; Lawton, 2010). Generally, in fact, during a complex environment exploration requiring a prolonged self-motion, it is more efficient to maintain an allocentric map of the environment than to continuously update multiple egocentric representations. However, the translation between egocentric and allocentric information is not equal: it is much more difficult to translate from egocentric to allocentric information than vice versa. The translation between these two different systems of coordinates that requires translating action-oriented egocentric representations into allocentric representations (e.g., the body references right/left become environmental references north/south) determines a cost in terms of time (Iaria et al., 2003; Etchamendy and Bohbot, 2007; Iglói et al., 2009). However, data demonstrate that individuals are nevertheless able to switch from one representation to another also during navigation, suggesting that people have both egocentric and allocentric coordinates at their disposal, although it is generally agreed that allocentric representations are more cognitive demanding than egocentric ones. This is also supported by Siegel and White's model (1975) that suggests a cumulative and hierarchical organization of spatial knowledge with high-level stages encompassing features of the lower stages. For these authors, in order to have an allocentric representation of the environment it is necessary to have acquired the egocentric representation. This seminal model supports our results that demonstrate how learning and retrieving a path is easier through an egocentric frame of reference. Indeed, from this point of view, the egocentric reference is the first system that humans develop to move through the environment. The importance of starting from an egocentric first-person perspective representation is also assumed in the animal models of spatial navigation (O'Keefe and Nadel, 1979). Egocentric navigation, in fact, resembles Taxon navigation (O'Keefe and Nadel, 1979), whereas allocentric navigation resembles Place navigation (O'Keefe and Nadel, 1979). Moreover, Byrne and Becker (2008) focus on the fact that spatial information from the environment must reach the brain via sensory receptors in an inherently egocentric sensory representation.

To summarize, gender differences in spatial navigation performance are reduced when participants become familiar with the environment. In our case, the familiarity was acquired through the time of exposure to the map or the number of repetitions performed to learn the path. With a high level of familiarity participants are able to perform spatial tasks with a high cognitive load, such as the translating from one frame of reference to another. In general, participants show a preference and a better proficiency when learning occurs in egocentric frames of reference. Overall, these results indicate that interactions between environmental demands and cognitive processes compensate for gender differences in spatial navigation performance.

AUTHOR CONTRIBUTIONS

RN and LP: made substantial contributions to conception and design, participated in drafting the article, or revising it critically for important intellectual content; AM, MG, AR, and OA: made contributions to acquisition of data, and analysis and interpretation of data; CG: gave contributions in interpretation of data; final approval of the version to be submitted and any revised version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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